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OXYGEN CONCENTRATION AND BIOLUMINESCENCE INTENSITY

I. BACTERIA AND FUNGI

J. WOODLAND HASTINGS[1]

Department of Biology, Princeton University, New Jersey
and the Marine Biological Laboratory, Woods Hole, Massachusetts

SIX FIGURES

INTRODUCTION

Although bioluminescence occurs in a sporadic way in over 40 orders of animals, it has been possible to demonstrate the luminescent system *in vitro* in only 5 orders of some 21 groups investigated.[2] In these 5 it has been shown that the reaction proceeds only in the presence of at least three substances, an enzyme, luciferase, its substrate, luciferin, and molecular oxygen. Of the other forms, where it has not been possible to separate luciferin and luciferase from the intact cell, all but a very few may be shown to require oxygen for the production of light.[3] It must be borne in mind, however, that the fact that light emission ceases in the absence of oxygen in the intact organism does not necessarily indicate that oxygen is utilized in the luminescent reactions. The anaerobic conditions might be interfering with certain cellular functions upon which the luminescent system is dependent. Evidence that this is not the case, and that oxygen is in fact utilized in the luminescent reactions in bacteria, was given in the work of Johnson, van

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[2] For a general treatment of bioluminescence, see Harvey ('40, '52).

[3] Very little is known about these exceptional cases in which extracts will luminesce in the complete absence of dissolved oxygen (see Harvey, '26; Harvey and Korr, '38).

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Schouwenburg and van der Burg ('39). They have shown that a substance, assumed to be luciferin, may be formed under anaerobic conditions. In spite of the uncertainty, it is generally assumed by investigators that the luminescent reactions in bacteria and fungi involve a luciferin-luciferase system similar to that where the compounds have been separated.

There have been few previous studies concerned with the role of oxygen in the reactions in bioluminescence. Luminous bacteria have been long known to maintain luminescence at very low oxygen concentrations. Shoup ('29) studied the respiration of luminous bacteria *(A. fischeri)* as a function of oxygen tension and noted that the light intensity began to decrease only after the respiration had been lowered by 50%, at 0.25% oxygen. Shapiro ('34), in a study of the relation between light intensity and oxygen concentration, using a fresh water species of bacteria, found that the intensity was unimpaired until the oxygen concentration reached 0.14%. Below this concentration the intensity fell off rapidly and approached zero at 0.01%. The data show considerable scattering, and it is not unlikely that secondary effects of low oxygen concentrations produced deleterious effects. Harvey and Morrison ('23) determined that light was just visible when hydrogen containing 1 in 10^5 parts of oxygen was bubbled through a dilute suspension of luminous bacteria. More recently Meyer ('42) reported that 1 in 10^{10} parts of oxygen is sufficient to give visible luminescence in bacteria.

If, as is assumed, oxygen participates as a reactant in the luminescent reactions, it would be expected that the light intensity should be related to the oxygen concentration by a definite function. A further and more quantitative study of this relation in several luminous organisms would therefore help in understanding the reactions in bioluminescence. The present paper provides information concerning the effect of varying oxygen concentration upon the light intensity in a bacterium *(A. fischeri)* and three species of luminous fungi *(Panus stipticus, Armillaria mellea,* and *Clitocybe illudens).* Similar studies on organisms such as *Cypridina hilgendorfii,* where the photogenic substances have been separated from the organism and partially purified will be published subsequently.

THEORY

Numerous schemes have been proposed for the reactions in bioluminescence. One of these, derived by Chance, Harvey, Johnson and Millikan ('40), from studies of the kinetics of the "flash" in bacteria and Cypridina, is essentially a combination of the earlier schemes of Harvey ('35) and Johnson, van Schuowenburg and van der Burg ('39). According to this scheme

$$LH_2 + A \rightarrow LH_2 \cdot A \qquad (1)$$
$$LH_2 \cdot A + \tfrac{1}{2}O_2 \rightarrow LH_2 \cdot A \cdot O \qquad (2)$$
$$LH_2 \cdot A \cdot O \rightarrow A' + H_2O + L \qquad (3)$$
$$A' \rightarrow A + h\nu \qquad (4)$$

In the reaction of Cypridina luciferin (LH_2), luciferase (A), and oxygen, these authors discovered that when luciferin and luciferase are mixed anaerobically and subsequently mixed with oxygen, the reaction is considerably faster than when luciferin and luciferase are mixed aerobically. Stated otherwise, $(LH_2 + A) \times (O_2)$ is faster than $(LH_2 + O_2) \times (A + O_2)$. The combination of luciferin with the luciferase was thus written as occurring first, followed by the combination of oxygen with the luciferin-luciferase complex ($LH_2 \cdot A$).

As it was found by the above authors that the kinetics of the flash in both *Cypridina* and bacteria were independent of oxygen pressure between 380 and 2 mm of oxygen, it was assumed that the combination with oxygen was a very fast reaction, being essentially complete before any appreciable amount of the complex had broken down to produce light.

Inasmuch as they also found that the light intensity during the flash takes a finite time to attain its maximum, the breakdown of the luciferin-luciferase-oxygen complex must proceed through more than one step. The kinetic data are satisfied mathematically by the assumption that the breakdown occurs via two consecutive monomolecular reactions. The excited molecule (A′), which is formed in the first step, subsequently breaks down with the emission of a quantum of light. It should be pointed out that the designation of the luciferase as the light emitting molecule is, from our present knowledge, an arbitrary choice.

A satisfactory description of the relation between oxygen concentration and light intensity may be made if the above scheme is modified to some extent. First of all it is assumed that one molecule of oxygen in lieu of $\frac{1}{2}O_2$ reacts; secondly, reactions (1) and (2) are considered to be thermodynamically reversible; lastly it is not supposed that luciferin must react with luciferase before oxygen enters the reaction. The luciferase is viewed as having two types of active sites, one for the adsorption of oxygen, the second for the adsorption of the luciferin. In order for the reaction to occur it is supposed that the two substances must be present simultaneously on their respective sites. Thus

$$LH_2 + O_2 \xrightleftharpoons{\text{(luciferase, A)}} LH_2 \cdot A \cdot O_2 \qquad (5)$$

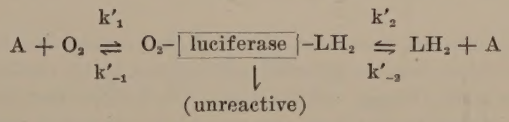
$$LH_2 \cdot A \cdot O_2 \rightarrow C^* + P + A \qquad (6)$$

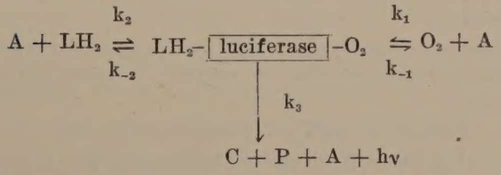
$$C^* \rightarrow C + h\nu \qquad (7)$$

where C* represents the excited molecule resulting from the breakdown of the complex, A the regenerated luciferase, and P the other products of the breakdown of the complex. This interpretation is consistent, moreover, with the findings of Chance et al. ('40) discussed above. In particular one is not obliged to consider that the luciferase must combine with the substrates in a given sequence; it is sufficient to say that the velocity constant, k_1, for the combination of the oxygen with the luciferase is greater than k_2, for the combination of the luciferin.

It can be shown that, if the above mechanism were holding, the relation between either oxygen or luciferin concentration and light intensity should have the form of a rectangular hyperbola, approaching a limiting intensity at high concentrations of either substrate. In the present experiments with bacteria and fungi it has been found that this type of relation exists at relatively low oxygen concentrations (figs. 3 and 6).

At high oxygen concentrations, however, the light intensity passes through a maximum and decreases, instead of approaching a limiting value. In the *Cypridina* system Chase ('49) has shown that the variation of light intensity with luciferin concentration also has the form of a rectangular hyperbola. High luciferin concentrations, however, tend to inactivate the luciferase (Chase, unpublished personal communication). One possible explanation for this decrease in light intensity at high concentrations of either substrate is that a competitive adsorption of the substrates occurs (see Laidler and Socquet, '50a). In this case it is supposed that oxygen and luciferin are not adsorbed exclusively on their respective sites; oxygen may be adsorbed to some extent on the sites normally reserved for luciferin and conversely luciferin on the oxygen sites. The dissociation constants for the adsorption of oxygen and luciferin on the foreign sites are taken to be such that the adsorption is negligible at low concentrations of either substrate. At higher concentrations, however, the competitive adsorption becomes appreciable and results in inhibition, since the light reaction occurs only when oxygen and luciferin are adsorbed simultaneously on their specific sites. Schematically this is represented below, showing the velocity constants for the combination of oxygen and luciferin on both specific and foreign sites; the left side of the luciferase is represented as specific for luciferin and the right side for oxygen. The overall velocity constant for the breakdown of the complex is again k_3.


$$\begin{array}{c} A + O_2 \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} O_2\text{–}\boxed{\text{luciferase}}\text{–}LH_2 \underset{k'_{-2}}{\overset{k'_2}{\leftrightharpoons}} LH_2 + A \\ \downarrow \\ \text{(unreactive)} \end{array} \qquad (8)$$


$$\begin{array}{c} A + LH_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} LH_2\text{–}\boxed{\text{luciferase}}\text{–}O_2 \underset{k_{-1}}{\overset{k_1}{\leftrightharpoons}} O_2 + A \\ \Big\downarrow k_3 \\ C + P + A + h\nu \end{array} \qquad (9)$$

The luciferase molecules with either oxygen or luciferin adsorbed on both sites are also unreactive.

On the basis of the above scheme the expected relation between light intensity and concentration of oxygen or luciferin may be given. If θ_1 is the fraction of oxygen sites covered by oxygen molecules, and θ'_1 the fraction of oxygen sites covered by luciferin molecules, and K_1 and K'_1 the corresponding equilibrium constants, it follows that $(1 - \theta_1 - \theta'_1)$ is the fraction of bare oxygen sites. The rate, v_1, at which the combination of oxygen on the oxygen sites occurs is proportional to the concentration of oxygen and the fraction of luciferase molecules with bare oxygen sites, or,[4]

$$v_1 = k_1 [O_2] (1 - \theta_1 - \theta'_1) \tag{1}$$

while the rate, v_{-1}, at which the dissociation of oxygen from the complex occurs is a function of the number of complexes

$$v_{-1} = k_{-1}(\theta_1) \tag{2}$$

At equilibrium $v = v_{-1}$ so that

$$k_1 [O_2] (1 - \theta_1 - \theta'_1) = k_{-1} (\theta_1) \tag{3}$$

and

$$\frac{(\theta_1)}{(1 - \theta_1 - \theta'_1)} = \frac{k_1}{k_{-1}} [O_2] = K_1 [O_2] \tag{4}$$

in a precisely similar manner, for the adsorption of luciferin on the oxygen sites, we may write [5]

$$\frac{(\theta'_1)}{(1 - \theta_1 - \theta'_1)} = K'_1 [LH_2] \tag{5}$$

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[4] This is the equilibrium treatment, based on the assumption that the complex $LH_2 \cdot A \cdot O_2$ lies at a low free energy level with respect to its products. It has been shown for similar cases (Briggs and Haldane, '23; Laidler and Socquet, '50) that a steady state treatment results in an equation which predicts a similar relationship between the variables.

[5] The luciferin concentration at equilibrium is less than the initial concentration, $[LH_2]_0$, by the amount combined with the enzyme. A more exact treatment would make the substitution

$$[LH_2] = [LH_2]_0 - [A]_0 \theta_2 - [A]_0 \theta'_1$$

This may be omitted if the enzyme concentration is low. A further reason for omitting the substitution here is the fact that it is quite unlikely that the luciferin concentration in the living organism is controlled only by its equilibrium with the luciferase and oxygen (see Discussion).

Solving equations (4) and (5) for θ_1

$$\theta_1 = \frac{K_1 [O_2]}{1 + K_1 [O_2] + K'_1 [LH_2]} \qquad (6)$$

Similarly the fraction of luciferin sites covered with luciferin is

$$\theta_2 = \frac{K_2 [LH_2]}{1 + K_2 [LH_2] + K'_2 [O_2]} \qquad (7)$$

The light intensity is proportional to the concentration of ternary complexes, $[LH_2 \cdot A \cdot O_2]$, where oxygen and luciferin are simultaneously adsorbed on their specific sites. As the concentration of ternary complexes is given by $[A]\ \theta_1\theta_2$, we have

$$I = \frac{k_3 K_1 K_2 [A] [LH_2] [O_2]}{(1 + K_1 [O_2] + K'_1 [LH_2]) (1 + K_2 [LH_2] + K'_2 [O_2])} \qquad (8)$$

Since at low oxygen concentrations the term $K'_2 [O_2]$ may be neglected, the relation between light intensity and oxygen concentration in that region may be described by

$$I = \frac{I_{max} [O_2]}{K_s + [O_2]} \qquad (9)$$

where

$$I_{max} = \frac{k_3 K_2 [A] [LH_2]}{1 + K_2 [LH_2] + K'_2 [O_2]} \qquad (10)$$

and

$$K_s = \frac{1 + K'_1 [LH_2]}{K_1} \qquad (11)$$

At higher oxygen concentrations where the term $K'_2 [O_2]$ is appreciable, it can be seen from equation (8) that the light intensity will decline as the oxygen concentration is increased.

Equation (9) is the usual form of the Michaelis-Menten relation, but Lineweaver and Burk ('34) have shown that a more convenient form may be had if its reciprocal is multiplied by $[O_2]$ so that

$$\frac{[O_2]}{I} = \frac{[O_2]}{I_{max}} + \frac{K_s}{I_{max}} \qquad (12)$$

If the equation is satisfied, a plot of $[O_2]/I$ against $[O_2]$ should result in a straight line. The maximum velocity of the reaction, I_{max}, is given by the reciprocal of the slope of the resulting line, and from the intercept, equal to K_s/I_{max}, the value of K_s may be found.

While it cannot be shown that all of the assumptions upon which the above theory is based are necessarily holding in the living organisms (see Discussion), at the same time such a formulation leads to a very satisfactory and consistent interpretation of the experimental results.

METHODS

Due to the low light intensity of the luminous fungi, as well as to the desirability of using extremely dilute suspensions of luminous bacteria, the vacuum multiplier type phototube was used for measurement. A careful calibration of each tube and the associated circuits is imperative, however, due to the variance in characteristics from tube to tube. In calibrating the tubes the light flux was varied in a known way by changing the angle between two polaroid sheets. A phototube (RCA 931-A) with a low dark current and low fatigue characteristics was selected, whose response to light flux was linear over the range of light levels used. Under conditions of high humidity the dark current became excessively high due to ohmic leakage, necessitating sealing the tube in a chamber with a drying agent.

Gas mixtures containing oxygen concentrations ranging from 0.001% to 100% were prepared with apparatus constructed especially for the purpose, shown schematically in figure 1. Hydrogen, oxygen and carbon dioxide were obtained from commercial tanks, and air from a compressor. The tanks were attached to the input side of the appropriate flow meters, with mercury overflow valves adjusted to the proper pressure. The flow meters were constructed entirely of glass, and the pressure drop across the capillary, measured by the difference in the level of the water in the manometer, gave the rate of flow of the particular gas passing through. The water in the manometer was equilibrated with the particular gas prior to each experiment by means of a tonometer built into one arm of the manometer. All connections between glass parts

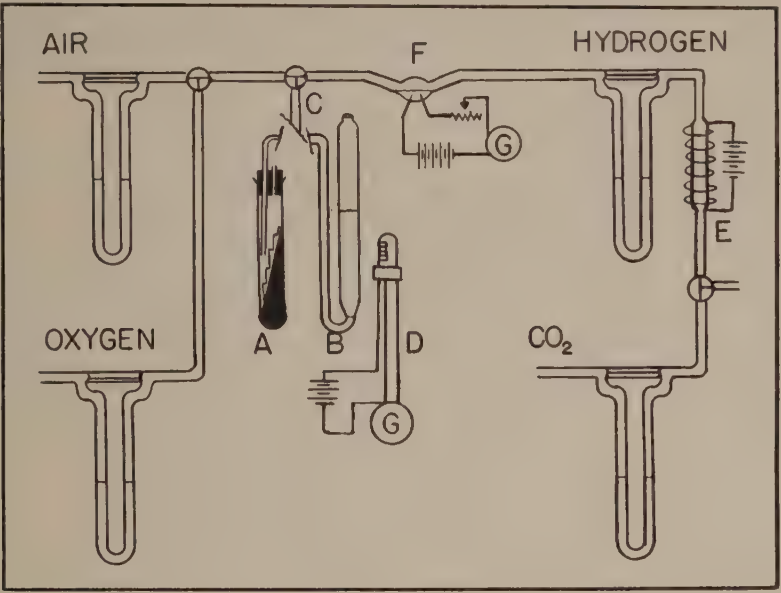

Fig. 1 Schematic diagram of the apparatus used in the preparation of the gas mixtures. Above each of the flowmeters is written the gas used with it; mixing of the gases occurred at the three way stopcocks.

A. An agar slant with fungus mycelium on the surface, showing the glass tube for passing gas mixtures through the test tube.

B. The type of vessel used in experiments with bacterial suspensions. A sintered glass disc in the base divides the gas stream into very fine bubbles as it enters the tube.

C. Lead tubing containing gas mixture which is connected to either tube A or B.

D. Photomultiplier tube and associated circuits.

E. Furnace with platinized asbestos for removing last traces of oxygen from the gas flow.

F. Electrolytic chamber with 3% KOH solution. Oxygen is liberated at one electrode with the passage of current.

G. Milliammeters.

were made with lead tubing sealed with de Khotinsky cement so that no leakage or diffusion of gases could occur.

Gas mixtures between 21% and 100% oxygen were prepared by mixing air and oxygen at the three way stopcock at the left of figure 1. Mixtures between 0.5% and 21% oxygen resulted from mixing air with hydrogen purified over hot platinized asbestos at the stopcock with the lead at C. The method of Meyer ('42) was adopted for preparing gas mixtures from 0.001% to 1.0% oxygen. A 3% solution of KOH was placed in the chamber (F) with platinum electrodes, where oxygen and hydrogen are liberated with the passage of current. The amount of electrolysis current was regulated with a decade resistance box and measured with a milliammeter. From Faraday's law the rate of oxygen evolution may be calculated, and when combined with the determination of the rate of flow of the purified hydrogen gives the percentage of oxygen present in the gas flow. Over the range where this method overlapped with the direct mixing, the two gave identical results.

Carbon dioxide was mixed with hydrogen prior to the point where it passed over the platinized asbestos in order to assure the removal of any oxygen present. A correction for the effect of carbon dioxide on the hydrogen flow meter was applied. The final gas mixture was led into the vessel containing the experimental material through the tube at (C).

Vessel (A) is a test tube containing a fungus mycelium on the surface of an agar slant with the appropriate connections for passing the gas mixtures over the surface. Under the experimental conditions used the gas was replaced every 6 to 8 seconds. From 20 to 30 seconds were required to extinguish the luminescence with pure hydrogen flowing.

Vessel (B) is the type used in the studies with the bacteria, where the liquid phase must be equilibrated with the gas mixture being introduced. The gas entered the vessel at its base through a built in sintered glass disc. The stream was thus very effectively divided into very fine bubbles which rapidly brought the tension of gases in solution into equilibrium with those in the gas phase. The flow rate with this vessel was maintained at about 170 ml per minute, and about 45 seconds were required for complete deaeration with pure hydrogen. The tube was covered with black tape except for a small window opposite the phototube at a point where the fine bubbles were flowing regularly, so that intensity fluctuations due to bubbling were not encountered.

FUNGI

The luminous diploid mycelium of each of the three species was grown upon slants of malt agar inoculated from two to 4 weeks before an experiment. The cultures were maintained in a dark room at room temperature (23–25°C.), and were chosen for experiments (also at room temperature) when luminosity was well developed. The data and discussion which follow refer to experiments carried out using the fungus, *Panus stipticus,* unless otherwise noted. The greater part of the fungus growth is in the air, and the gas comes in immediate contact with the hyphae.

At all oxygen concentrations below that of air the intensity of luminescence is decreased. An individual value was obtained in the following manner. Air was passed over the fungus until the intensity had remained constant for a period of 5 minutes or more. Then a mixture of hydrogen and air with a lowered oxygen concentration was passed over the fungus and the changes in light intensity recorded for a period of two minutes. Air was then reintroduced and the changes recorded. When the intensity returned to its original value in air, the same procedure was repeated using a different oxygen concentration.

A typical plot of light intensity changes during such a procedure is shown in figure 2, where the gas mixture contained 6.6% oxygen. After the initial rapid decrease, the light intensity increased considerably during the first 20–30 seconds. Though part of this effect may possibly be due to pressure equilibration in the different flow meters, such an explanation cannot account for the values of the magnitude or time parameters involved. If in fact this effect were due to the initial mixing of the gases, it should be experimentally possible to demonstrate the opposite effect (i.e., approaching the plateau

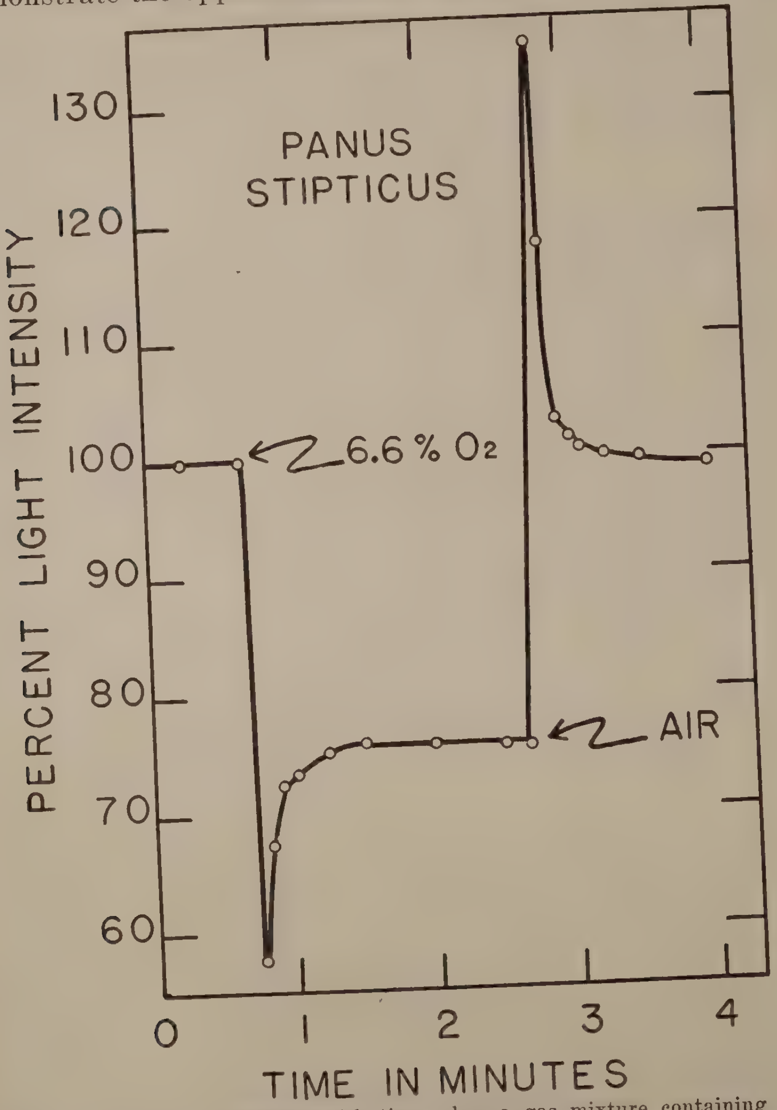

Fig. 2 Changes in intensity with time when a gas mixture containing 6.6% oxygen is passed over the fungus *Panus stipticus* and then returned to air. The maximum and minimum points were observed directly. The original intensity in air is considered as 100%.

from the other direction), by reversing the relative pressures in the flow meters. Such a procedure gave essentially the same result as shown in figure 2. Assuming then that this increase is real, it is possible to understand how, under these conditions, the concentration of either luciferase or the luciferin-luciferase complex might increase. If the luciferase were poisoned by oxygen to an appreciable extent in air, then upon lowering the oxygen concentration it would be expected that some inactive luciferase molecules would be released and thus increase the concentration. It has been assumed, however, that the poisoning is negligible in air. It seems more likely that, since the concentration of the luciferin-luciferase complex would be expected to be greater when in equilibrium with a lower oxygen concentration, the rise represents the transient in the reaction velocity resulting from the concentration change of the complex.

It can be seen from figure 2 that the intensity remains essentially constant after the first 30–40 seconds. This was true at all oxygen concentrations, and the values for light intensity were arbitrarily taken 80 seconds following the minimum in the dip. The original value in air was considered as 100% intensity.

A plot of these values in terms of $[O_2]/I$ against $[O_2]$ (from equation 12) is shown in figure 3B. The white and black circles are data from two different cultures (white circles, culture 1; black circles, culture 2). Two straight lines, differing both in their slopes and intercepts, describe the points well. From the slope the value for the maximum intensity may be calculated. This turns out to be 19.5% above the intensity in air for culture 1 and 38.0% for culture 2. The values plotted in figure 3A (same symbols) were obtained by recalculating the values for the light intensity, using as the 100% intensity the calculated maximum value in place of the intensity in air. Two rectangular hyperbolas with different constants result.

When oxygen concentrations higher than 21% are passed over the fungus, it is found that the intensity during the first few seconds is in fact greater than in air. Figure 4 shows the changes in light intensity when 100% oxygen was passed over culture 1. The increase is seen to be about 20% above the intensity in air, closely corresponding to the calculated value of 19.5%. The similar increase for culture 2 was 39.5% (calculated value, 38.0%). The intensity increase is transitory, how-

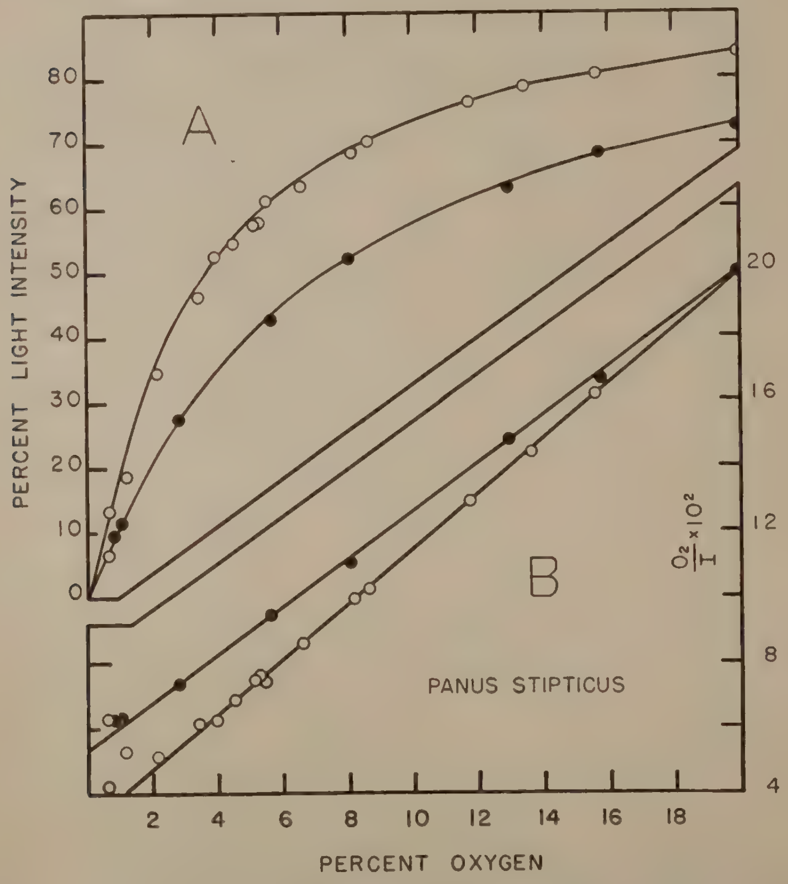

Fig. 3 Plot of the data from two different cultures of *Panus stipticus*. White circles, culture 1; black circles, culture 2.

B. Plot of the data according to equation (12) of text. $[O_2]$ is expressed in per cent oxygen and I in per cent light intensity in air. From the slopes of the lines the maximum intensity may be calculated.

A. Plot of per cent light intensity against per cent oxygen concentration. One hundred per cent light intensity is the value calculated from the slopes in plot 3 B, instead of the value in air.

ever, and a decay in intensity occurs subsequent to it, as long as the flow of 100% oxygen is maintained. When returned to air, a sudden drop in intensity is observed, followed by a reversal of the decay which had occurred. Oxygen concentrations between 21% and 100% gave effects of a similar nature to those shown but of a smaller magnitude. Between 21% and 60% oxygen the intensity remained above that in air; up to about 85% oxygen the intensity was less than 5% below that in air, while at higher concentrations the inhibitory effect became pronounced.

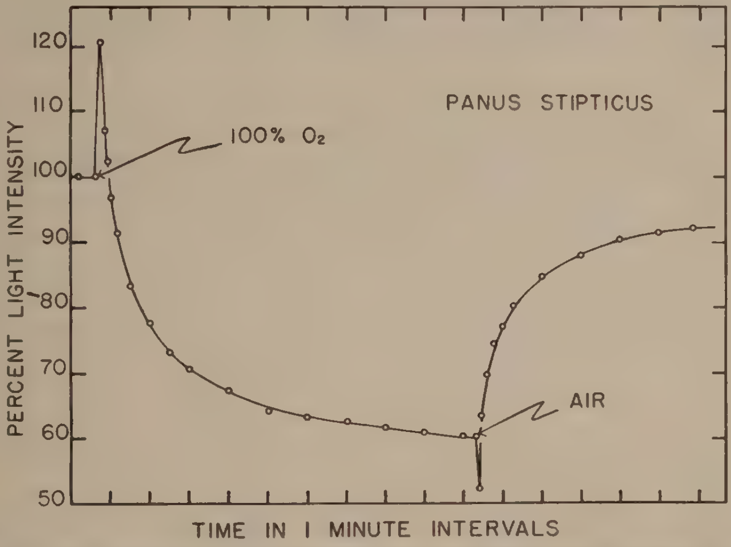

Fig. 4 The changes in light intensity with time when 100% oxygen is passed over the fungus (culture 1) for ten minutes and then returned to air. The initial increase of 20% corresponds to the value for the maximum intensity calculated from the slope in figure 3 B.

The intensity changes in high oxygen concentrations may be interpreted in terms of competitive adsorption. While the initial effect of 100% oxygen is to increase instantaneously the velocity of the reaction to the value predicted by the equation, there is quite rapidly an inhibitory effect. This is interpreted as being the result of the adsorption of the oxygen molecules on the luciferase sites normally reserved for the luciferin. This effectively immobilizes a proportion of the luciferase molecules; when the fungus is returned to air the reversal of the process occurs.

In figure 2 the prominent flash of luminescence following the period of partial anaerobiosis is seen. This occurred following exposure to all oxygen concentrations below 21%. The intensity of this flash is dependent upon the concentration of oxygen during the two minute period of partial anaerobiosis.

TABLE 1

Panus stipticus, culture 1.

The intensity of the flash upon admission of air following a two minute period of exposure to a reduced oxygen concentration. The values were not significantly different with different times of partial anaerobiosis.

% OXYGEN	FLASH INTENSITY %	% OXYGEN	FLASH INTENSITY %
0.64	220	5.27	151
0.68	233	5.46	150
1.42	205	6.6	135
2.15	210	8.18	131
3.42	164	8.65	129
3.95	168	11.75	114
4.53	166	13.63	108
5.17	149	15.60	107

Table 1 shows how the intensity of the flash is greater after exposure to progressively lower concentrations of oxygen.

Under pure hydrogen, flashes of the magnitude obtained in 0.68% oxygen (table 1) were never obtained. Figure 5 shows a series of flashes obtained after $\frac{3}{4}$, $1\frac{1}{2}$, 3, 5, 9, 16, and 31 minutes exposure to pure hydrogen. At three minutes it can be noted that the form of the decay curve becomes somewhat irregular, and that the area under the curve is increased. At 5 minutes, with the flash reduced considerably, the apparent cause of this irregularity becomes obvious. This new feature, which we will call the secondary rise (see Johnson et al., '39), becomes increasingly prominent with increased time of exposure to hydrogen. The secondary rise always occurs subsequent to a flash (even though the height of the flash may be less than the original intensity in air), and is represented by a relatively slow rise in light intensity, whose magnitude increases with increasing time of anaerobiosis. The intensity

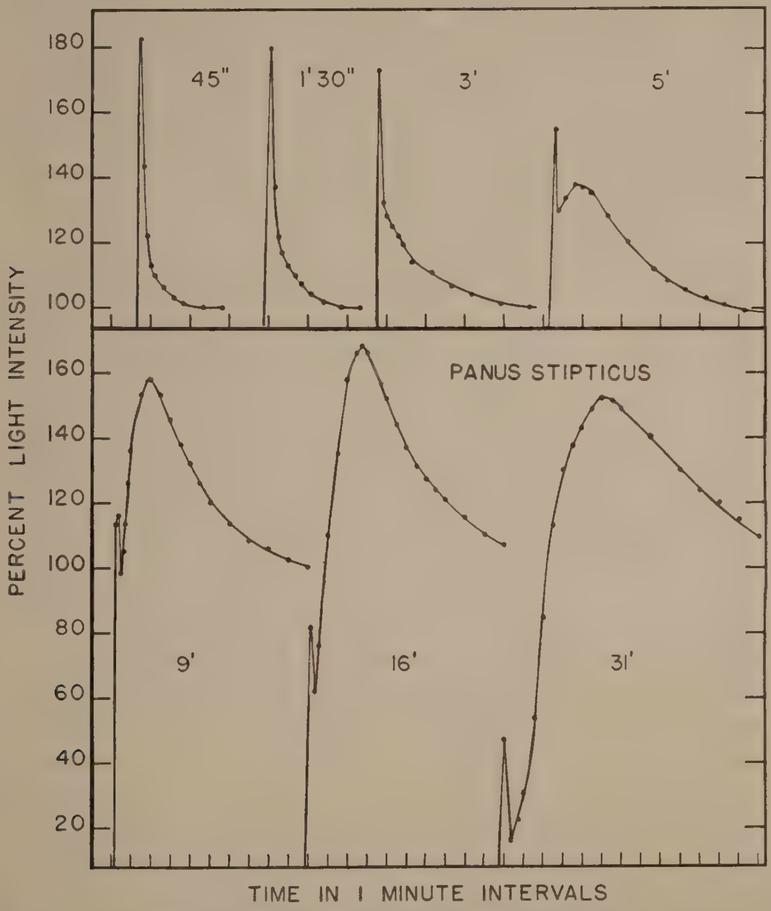

Fig. 5 The changes in light intensity which occur when air is admitted after anaerobiosis. The time the culture was kept under hydrogen is indicated above each graph. Intensity is given in terms of the per cent of the intensity in air before anaerobiosis. Note that the time axis is reduced by one-half in the lower part. Maximum and minimum values were observed directly.

levels off at the original value in air following the secondary rise.

Although the experimental methods necessary to give quantitative data concerning the detailed character of the flash were not available, it can be stated that the time necessary for the flash to reach its peak is of the order of seconds at room temperature. It is of interest to compare this value with that for the bacteria, 0.34 seconds, and in turn with the value for *Cypridina*, 0.007 seconds (Chance, Harvey, Johnson and Millikan, '40), also at room temperature.

The general characteristics of the flash and secondary rise described above are somewhat similar to corresponding changes described by Johnson, van Schouwenburg and van der Burg ('39) in luminous bacteria. These authors ascribed the flash to an accumulation of luciferin during anaerobiosis, while the reversible decrease after longer periods of anaerobiosis was interpreted as being the result of proteolytic hydrolysis of luciferase within the cells in the absence of oxygen. The secondary rise then represented the slow reformation of luciferase in the presence of oxygen. In the light of the present experiments, a more consistent interpretation of the effects of partial or complete anaerobiosis may be made on the basis of the two substrate system. First of all, it does not appear sure that the flash results from an accumulation of luciferin, since it has been shown (Amberson, '22; Chance et al., '40) that the flash occurs also in *Cypridina* extracts where no luciferin production is taking place. An alternative interpretation may be made upon the assumption (referred to previously) that the concentration of the luciferin-luciferase complex varies with the oxygen concentration with which it is in equilibrium. At progressively lower oxygen concentrations the flash, upon the admission of oxygen, becomes greater (table 1). It is significant to note that this relationship was found to be independent of time within rather wide limits (i.e., the intensity of the flash after 4 minutes under 6.6% oxygen was the same as after two minutes).

The reversible decrease which appears following anaerobiosis appears to be a result of the inactivation of luciferase, since it is not likely that the luciferin concentration has decreased during this time. On the contrary, it appears that the production of luciferin, or some luciferin precursor, continues during anaerobiosis, since the total amount of light produced increases following longer periods of anaerobiosis. The inactivation of luciferase may be accounted for if it is assumed that during anaerobiosis the luciferin is adsorbed on the sites normally reserved for oxygen. With the subsequent admission of oxygen this inactivation is reversed through the utilization of luciferin and the competition of oxygen for the sites; that the intensity passes through a maximum (secondary rise) considerably above the steady state intensity is to be expected, since it is clear from the flash that the reaction rate is not at its maximum with respect to luciferin concentration. The theory would predict that as the luciferin concentration was increased the intensity would pass through a maximum.[6] The secondary rise results from an initially high luciferin concentration; as the luciferin is used up the intensity passes through this maximum in the reverse direction.

When the data obtained for the three species of fungi were compared, it was found that in all experiments the relation between light intensity and oxygen concentration could be described by a rectangular hyperbola. Different constants, however, were to be associated with the curves from different species (see table 2). Paralleling these differences in the oxygen-intensity curve were quantitative differences in the appearance of the flash and secondary rise. However, a second and much younger culture of *Panus* (culture 2) was found to differ from culture 1 by a greater degree than had either of the other two species. It was therefore concluded that the differences observed between species were not a result of species differences, but rather a result of the condition of the fungus itself. It was significant however that with any one culture the experiments could be repeated within a period of a few days with the same results.

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[6] This has been observed in the isolated Cypridina system where luciferin concentration may be varied experimentally (Chase, personal communication).

BACTERIA

The experiments on bacteria were carried out with the marine luminous bacterium, *Achromobacter fischeri,* whose temperature optimum is between 26–29°C. The bacteria were grown on peptone agar slants containing 3% NaCl, 0.3% glycerol, and 0.3% $CaCO_3$ (Farghaly, '50). Maximum luminescence was attained between 15 and 17 hours after inoculation, at which time the bacteria from a single test tube were removed and suspended in sterile 3% NaCl. The cells were then washed twice by centrifugation in 25 ml of NaCl. After the second washing the bacteria were resuspended in 100 ml of a solution containing equal parts 3% NaCl and M/4 phosphate buffer, pH 7.1 (referred to as "PN" by Johnson et al., '39). This suspension was then stored in the refrigerator at 2°C. and could be used as a stock solution for a period of at least 5 days. For an experiment, 1 ml of the stock solution was diluted to 100 ml in "PN." In experiments where substrate was added, 1 gm of dextrose per 100 ml of solution was added. All experiments were performed at room temperature, from 23 to 25°C.

TABLE 2

Summary of the data from three species of luminous fungi. Note that the variation between the three species is small compared to the second culture of Panus, which was a much younger culture than the others. See text for details.

FUNGUS	SLOPE	1/SLOPE	% INTENSITY IN 100% O_2	K_s GAS UNITS
Panus (culture 1)	0.84	1.19	120.5	3.70
Panus (culture 2)	0.73	1.38	138	7.45
Armillaria	0.86	1.16	116	3.36
Clitocybe	0.87	1.15	113	3.01

Although many investigators have found unwashed cells with added substrate to be suitable for experimentation, it was our experience that such preparations resulted in cells whose light emission with time was unpredictable. The intensity after one hour might decrease as much as 80% or in other cases might be 10% higher than at the outset. No clue as to the factors responsible for these irregularities was discovered. By using the above procedure for washing the cells, the course of luminescence over a time period was found to be quite reproducible, and the general results of Johnson's ('39) study upon washed cells were confirmed.

Washed cells stored in the refrigerator undergo a reversible decrease in intensity (measured at room temperature) during storage. When brought back to room temperature the intensity returns to approximately the original level over a period of from 40 to 80 minutes. The light intensity during an ensuing period of from one to three hours was found to vary less than 10%. In experiments where dextrose was added, the initial increase in intensity was somewhat greater, but the light intensity similarly remained approximately constant for at least one hour after reaching a maximum.

Thirty milliliters of a suspension such as described above were placed in a tube such as shown in figure 1 with a sintered glass disc in the base. When the intensity had become essentially constant, a gas mixture of low oxygen concentration was introduced. The changes in light intensity were then recorded for a period of two minutes, after which air was again bubbled through. The light intensity was then calculated as the percentage of that obtained in air. During the two minute period under a decreased oxygen concentration the intensity remained essentially constant at the lowered value. Upon returning to air the characteristic flash was observed, after which the intensity quickly returned to approximately the original level in air.

A plot of the per cent light intensity against oxygen concentration for three different experiments is shown in figure 6 A. With the aid of figure 6 B it can be seen that the points describe three rectangular hyperbolas, differing in the value of the constant, K_s, from equation (12). Table 3 shows the values for the slopes and constants of 13 experiments. It is significant to note that the data obtained in any one experiment give excellent agreement with the theoretical formulation, and that duplicate experiments on the same suspension

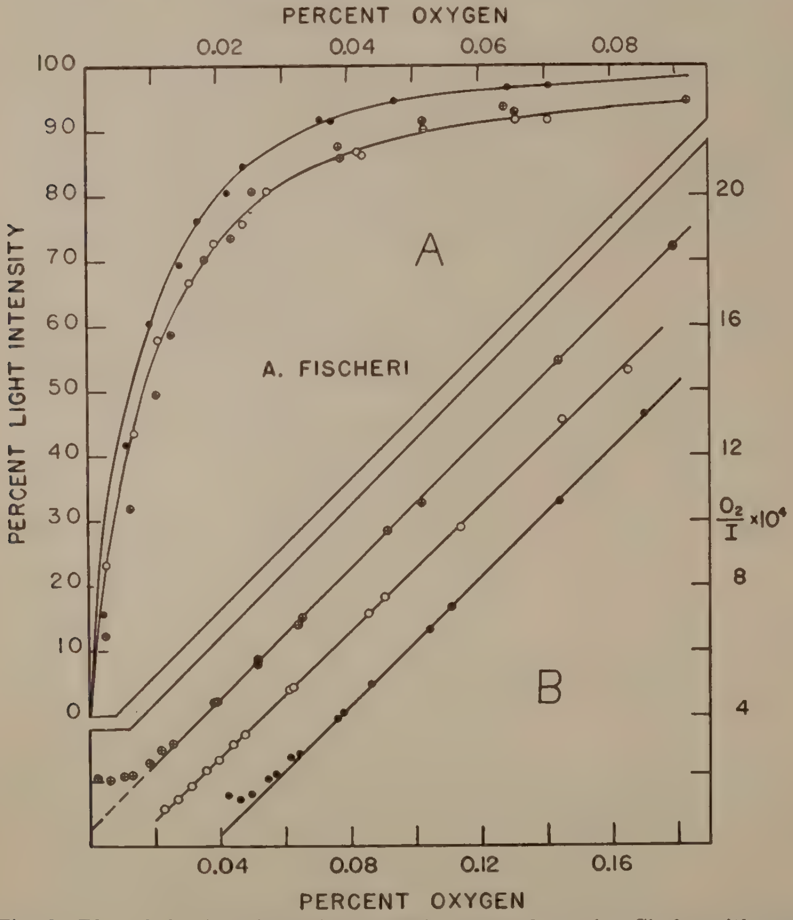

Fig. 6 Plot of the data from three experiments on bacteria. Circles with cross, experiment 1; white circles, experiment 1a; black circles, experiment 2 (see table 3). Note that the per cent oxygen axis is different on the two graphs. Carbon dioxide was added in experiment 1a.

A. Plot of per cent light intensity in air against per cent oxygen.

B. Plot of the data according to equation (12) of text. O_2 is expressed in per cent oxygen and I in per cent light intensity in air. The white and black circles have been shifted to the right along the horizontal axis by one and two divisions respectively to avoid confusion.

(indicated by letters following the numbers in table 3) give essentially identical results. The variations in the value of the constant in different experiments appear to be a result of changes taking place in the cells themselves, as all experimental procedures were repeated as exactly as possible. It was not found possible to determine the nature of these changes. Changes in the solubility of oxygen with temperature variations from experiment to experiment were too small to account for the differences in the values of the constant.

TABLE 3

Summary of 13 experimental runs on bacteria. Letters following the numbers represent duplicate runs on the same suspension. See text for details

EXPERIMENT NO.	SUBSTRATE	GAS	SLOPE	$K_s \times 10^3$ GAS UNITS
1	None	H_2	1.0	7.0
1a	None	$H_2 - CO_2$	0.96	7.5
2	None	H_2	1.0	2.5
3	None	H_2	1.0	3.0
3a	None	H_2	1.0	3.0
4	None	H_2	1.0	4.5
4a	None	H_2	1.0	4.5
5	1% Dextrose	H_2	1.06	5.0
6	1% Dextrose	H_2	1.0	5.0
6a	1% Dextrose	$H_2 - CO_2$	1.0	5.0
7	1% Dextrose	H_2	1.0	5.0
7a	1% Dextrose	$H_2 - CO_2$	1.0	5.0
8	1% Dextrose	H_2	1.0	2.7

When the data are plotted according to the linear equation (12), the slopes of the curves give the reciprocal of the maximum intensity. These slopes are found, in the case of the bacteria, to be unity or very close to it. As the intensities which were plotted were expressed in terms of the per cent of the intensity in air, the maximum rate of the reaction is identical with (100% × 1.0) the value in air.

The effect of 100% oxygen upon the intensity of luminescence was similar to what was observed for the fungi, with the exception that no transitory flash was observed. Upon bubbling 100% oxygen, the intensity began to decay immediately, and was reversible when air was subsequently bubbled through. The effect may again be understood in terms of the competitive adsorption of oxygen upon the luciferin sites on the luciferase.

One characteristic of *A. fischeri* which recommended its use in these experiments was its reported ability to withstand long periods of anaerobiosis with no deleterious result. Johnson, van Schouwenburg and van der Burg ('39) in their study of the flash, found that washed cell suspensions with added dextrose would immediately return to the original intensity (following the flash) after as long as 96 minutes under anaerobic conditions. If, on the other hand, dextrose was not added, a reversible decrease was observed following anaerobiosis. During one to two minutes following the admission of air the intensity returned to the level prior to the anaerobiosis. This was referred to as the secondary rise. These observations could not be repeated with the strain of *A. fischeri* which we used.[7] After 30 minutes' anaerobiosis the intensity following the flash was characteristically from 10 to 25% below the original level, and over an ensuing period of from 3 to 6 minutes returned to approximately the original level, often exceeding it somewhat. No difference between the substrate-free suspensions and those with added substrate was observed.

One clear difference between the experimental procedure of Johnson et al. and ours was that the carbon dioxide concentration was much greater under the conditions of their experiments. Several experiments were therefore run in which one portion of a suspension was deaerated with and maintained under hydrogen in the usual fashion, while a second was deaerated with a small amount (1%) of carbon dioxide added to the hydrogen. Whereas the suspension under pure hydrogen always showed the characteristic decrease, the suspension with carbon dioxide either showed no decrease or a decrease which was smaller in magnitude than the corresponding suspension under hydrogen. While the effect was evident, it was difficult to put on a quantitative basis.

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[7] Although this strain was obtained through the kindness of Professor F. H. Johnson, it is not certain that its origin is from the strain used by Johnson et al. ('39).

An experimental procedure which more clearly demonstrated this effect consisted in deaerating two suspensions with hydrogen, one of which had 1% carbon dioxide added to the stream. At the end of 30 minutes oxygen was added to the hydrogen streams so that the concentration in the gas flows was approximately 0.8%, well above the concentration normally needed for maximum luminescence. The light intensity in the suspension without carbon dioxide was found to be less than 50% of the original value, while the one to which carbon dioxide had been added returned immediately to the original intensity in air.

The effect was also clearly demonstrable in the data taken for the relation between oxygen concentration and light intensity. In experiments where hydrogen alone was used as the "carrier" gas there were significant deviations from the theoretical at low oxygen concentrations; when 1% carbon dioxide was added to the stream no such deviations were found (fig. 6 and table 3).

Thus under conditions of either very low oxygen concentration or complete anaerobiosis an apparent inactivation of luciferase may be observed. It may be supposed that this effect is also the result of the adsorption of luciferin on the oxygen sites, though there is no evidence that luciferin accumulates to any great extent in the bacteria. It is consistent, however, to find that the inactivation is quickly reversed in air, while a very low oxygen concentration (0.8%) is not sufficient to displace the adsorbed luciferin molecules. The mechanism by which carbon dioxide might prevent this effect is not clear. It is known (Farghaly, '50) that carbon dioxide is necessary for the growth of *A. fischeri*, and it has been shown (Loewy, '50) that carbon dioxide can prevent the destructive effects of anaerobiosis in a myxomycete. It does not seem likely that the mechanism which is responsible for the effect observed in the present experiments is similar to those previously reported.

DISCUSSION

The consideration of the luminescent system in bacteria and fungi on the basis of an enzymatic reaction involving two substrates has led to a very satisfactory and consistent interpretation of the results presented. Moreover, such a scheme is consistent with the results of previous work in the field. The luciferase is viewed as having two sites, one for the adsorption of oxygen and the second for the adsorption of luciferin. The reaction occurs only when these two substrates are present simultaneously on their specific sites. If competitive adsorption occurs, however, so that the substrates are not adsorbed exclusively on their own sites, it can be shown that the velocity of the reaction will pass through a maximum at high concentrations of either substrate. The fact, therefore, that high oxygen concentrations were found to decrease reversibly the light intensity is good evidence for the assumption of competitive adsorption in the two substrate system. The reversible decrease in light intensity observed after anaerobiosis in these organisms was interpreted as being the result of the adsorption of excess luciferin on the oxygen sites. In support of this there is good evidence that luciferin is accumulated anaerobically in the fungi. While there is no evidence that luciferin is accumulated anaerobically in the bacteria to any great extent, there is evidence that luciferin may be formed under anaerobic conditions (Johnson et al., '39).

Bioluminescence is ideally suited for kinetic studies, for the reaction velocity may be measured instantaneously and continuously (giving the rate of change of the reaction velocity as well). For example, in the case of the fungi the theoretical limiting velocity of the reaction may be calculated from the data taken at relatively low oxygen concentrations; by following the intensity continuously it is discovered that under 100% oxygen the limiting velocity is in fact reached momentarily. Whereas the adsorption of oxygen onto its specific sites is a very fast reaction (as shown also by Chance et al. ['40] for bacteria and *Cypridina)*, the adsorption of oxygen onto the luciferin sites is relatively slow.

However, since luciferin and luciferase have not been separated from the intact cell in the bacteria and fungi, it is impossible to evaluate the constants or to make a more precise analysis of the reactions. Because of this fact it is clear that the interpretation given is open to subsequent revision. For example, in equation (12) it was assumed (see footnote 5) that the concentration of luciferin remained constant while the oxygen concentration was varied. There is evidence, however, that indicates that the luciferin concentration might vary under different oxygen concentrations. The explanation of the flash was based on the assumption that the concentration of the luciferin-luciferase complex varied with the oxygen concentration. While this does not necessarily mean that the luciferin concentration changes under these conditions, it is not unlikely that it should. Furthermore, it is to be expected that the luciferin concentration in the living organism is also affected by its equilibrium with other reactions, both those which lead to its formation and possible side reactions. In spite of these uncertainties in making our assumptions it has been found that the results conform remarkably well to the theory. It must therefore be concluded that either (1) the errors resulting from the approximations in the assumptions are so small as to be insignificant in the mathematical formulation or (2) the mechanism of the reaction is a wholly different one which, however, leads to the same empirical relationship. For example, another possible explanation for the decrease in intensity at high oxygen concentrations which is wholly consistent is that luciferin undergoes a dark oxidation which becomes prominent at high oxygen concentrations, resulting in a decrease in light intensity. In support of this it is known that *Cypridina* luciferin is very readily oxidized without light emission. While it is clear that the interpretation given throughout the paper is a consistent one, it is impossible to ascertain the nature or magnitude of the uncertainties involved.

The fact that the values of the constant, K_s, were found to vary from one suspension or culture to another does not appear to be serious. Chase ('48) has shown in the partially purified *Cypridina* system how buffers and hydrogen ion concentrations can bring about such changes. It is not possible to say whether these factors or others are responsible in the present case, but it is not unlikely that the enzyme affinity should change from one culture to another.

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SUMMARY

A study was made of the relation between the intensity of luminescence and the oxygen concentration in a luminous bacterium and in three species of luminous fungi. In all organisms the light intensity increases hyperbolically as the oxygen concentration is increased, while at high oxygen concentrations the light intensity decreases from the maximum. Fifty per cent maximum luminescence occurs in the bacteria at 0.007–0.009% oxygen and in the fungi between 3 and 8% oxygen. A variation in the constant occurred in different cultures which was attributed to differences arising in the organisms.

Since both oxygen and luciferin react with the enzyme luciferase, the theoretical formulation for the relation between light intensity and oxygen concentration was based on the assumption that the enzyme contains two sites specific for the oxygen and luciferin, such that the reaction proceeds only when the two substrates are adsorbed on their respective

sites. It was shown that in such a two substrate system a competitive adsorption of the substrates, where one substrate competes for the site on the enzyme normally reserved for the second, and vice versa, will result in a decrease in the velocity of the reaction at high concentrations of either substrate. Such a reversible decrease in intensity was found to occur in both organisms at high oxygen concentrations. A reversible decrease in intensity was also found to occur following an extended period of anaerobiosis. This effect was attributed to the adsorption of luciferin on the oxygen sites, resulting in the reversible inactivation of the luciferase. It was found that in the bacteria this reversible inactivation of the luciferase did not occur if 1% carbon dioxide was added to the gas during anaerobiosis. The mechanism of this effect is not clear.

As there is no direct evidence that oxygen participates directly in the luminescent reactions of the intact cells of bacteria and fungi, the present study is considered as further indirect evidence for that assumption.

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A HISTOCHEMICAL STUDY OF FROMMANN'S STRIATIONS AND A QUALITATIVE DETERMINATION OF CHLORIDE IN MAMMALIAN NERVE FIBERS[1]

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SEVEN FIGURES

The significance of chloride in its effect on the respiration of nerve fibers (Chang et al., '35) and in propagation of the nervous impulse (Hodgkin and Katz, '49) has been considered. There is an indication that chloride ions may be necessary for the maintenance of the excitability of nerve fibers (Lorente de Nó, '47, p. 58). In view of these suggestions, it is of fundamental interest to determine if chloride is present in nerve and if it is present inside and/or outside of the axons.

Chloride has been shown to occur intracellularly in the giant axon of the squid (Bear and Schmitt, '39; Webb and Young, '40; Steinbach, '41). However, as yet, there is no direct evidence that chloride is present in mammalian axons. Amberson et al. ('38) report that there is a small indiffusible fraction of chloride in the sciatic nerve of the cat after the body chloride is replaced by perfusion with sulfates; this indiffusible fraction is presumably intracellular.

In axons, Frommann's striations can be produced by exposing to strong sunlight teased fresh mammalian nerve fibers previously treated with a solution of silver nitrate and nitric acid in the dark. Since this solution is a reagent for chloride, the Frommann's striations may serve as an indicator.

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[1] Preliminary Report: A. Hess, Anat. Rec., *109:* 303–304, 1951.

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Macallum and Menten ('06), who review thoroughly the literature of Frommann's striations, conclude that the striations are composed of silver chloride and that chloride is present in mammalian axons. Achard and Aynaud ('06) also believe that Frommann's striations are produced by silver impregnation of chloride. Macdonald ('07) accepts the results of Macallum and Menten and believes that chloride is present in the axon in a "masked" form. Noda and Yasuzumi ('30) state that it is the chloride of the axon which enters into the composition of Frommann's striations.

However, Fenn et al. ('34), Lison ('36) and Gersh ('38) have criticized severely Macallum's histochemical techniques and results. Lison believes that the silver nitrate-nitric acid reagent, employed by Macallum and other workers, is not specific for the identification and localization of chloride. He suggests that supplementary tests must be used for these purposes. Fenn et al. and Gersh consider chloride to be totally extracellular or interfibral. In addition, the theory that chloride occurs only extracellularly or that the "chloride space" is the same as the intercellular space (Fenn, '36; Höber, '45) has served to refute Macallum's conclusion.

In the present study, an attempt is made to demonstrate that chloride is present not only in the interfibral space, but it is present also in the axons. Use is made of the formation of Frommann's striations to indicate the presence and localization of chloride in nerve fibers. Tests are made on extract of nerve fibers and on extract of silver chloride produced in nerve fibers to supplement the identification of chloride. Evidence is to be presented that mammalian axons contain chloride, which enters into the formation of Frommann's striations.

MATERIAL AND METHODS

Throughout this study, sciatic nerves of white rats were used. The connective tissue sheath was removed quickly and the nerve fibers spread gently on a slide. For microscopic examination, the fibers were mounted in glycerine and covered with a coverslip.

Tests were performed both on nerve fibers and on extracts of nerve fibers. For the preparation of extracts, the teased specimen was lifted as a whole from the slide and placed in the appropriate reagent. The tests chosen were for the identification of chloride and silver chloride. All reagents used were tested for the presence of chloride and found to be chloride-free. All glassware and equipment were chemically clean before use.

RESULTS

Frommann's striations. Nerve fibers, spread on a slide and treated with a solution containing 1.7 gm silver nitrate and 1.5 ml nitric acid per 100 ml distilled water (hereafter referred to as the silver nitrate-nitric acid reagent) for 12 hours in the dark and subsequently exposed to strong sunlight, revealed striations in the axis cylinders (figs. 1–4). The striations usually started to form in the axon at the node of Ranvier, but might begin at an artificial rupture of the myelin sheath. The length of the striations and the distance between them usually increased as the distance from the point of entry of the silver solution into the axon increased. Other aspects of these striations are discussed elsewhere (Hess and Young, '51). It is not attempted here to discuss the physicochemical nature of the formation of Frommann's striations; Macallum and Menten ('06) discuss the striations of Frommann as an example of the Liesegang ring phenomenon of periodic precipitation.

Extraction of the substance responsible for the production of Frommann's striations in nerve fibers. Nerve fibers were spread on a slide and immersed in distilled water or in aqueous solutions of ammonia (33%), sugar (6%), potassium nitrate (1.5%), or potassium cyanide (5%) for 30 minutes. These fibers were then treated with the silver nitrate-nitric acid reagent in the dark for 12 hours. After subsquent exposure to strong sunlight, no striations of Frommann were revealed. Presumably these solutions could extract the substance responsible for the production of Frommann's striations in axons.

Identification of chloride in water extract of nerve fibers. From the foregoing, it is assumed that the substance responsible for the production of Frommann's striations in nerve fibers can be extracted with distilled water. Therefore, tests were performed on water extracts to determine if chloride is present.

Nerve fibers from a 4 cm stretch of the sciatic nerves were spread on a slide, then lifted off and placed in 5 ml distilled water for 12 hours. After this extraction, the nerve fibers were treated with the silver nitrate-nitric acid reagent for 12 hours in the dark and subsequently exposed to strong sunlight. No Frommann's striations or any trace of silver precipitate were evident.

The water extract appeared cloudy. The cloudiness was decreased considerably by adding a few drops of nitric acid. Upon adding the silver nitrate-nitric acid reagent, the extract became more turbid. A precipitate was obtained by centrifugation. The water extract also reacted with a 4% aqueous solution of mercurous nitrate (Treadwell and Hall, '32) or a 4% aqueous solution of lead acetate (Treadwell and Hall, '32) to yield precipitates.

These tests on the water extract of nerve fibers indicate that the substance responsible for production of Frommann's striations is soluble in water and that the extract contains a halide. To determine definitely that only chloride and not bromide or iodide is involved in the production of Frommann's striations in nerve fibers, further tests were performed as follows:

Twenty-five milliliters of the water extract from a total length of 10 cm of sciatic nerve fibers were evaporated in a retort. The residue was covered with powdered potassium dichromate and concentrated sulfuric acid. The mixture was heated gently. A light brown liquid distillate was collected. Since iodide does not give this reaction, it is, therefore, excluded. Under the experimental conditions, bromide, if present in the water extract, is oxidized to elementary bromine, which interferes with the diphenylcarbazide test (see below). To detect the presence of bromide in the water extract, phenol was added to the brown liquid distillate. No precipitation of tribromophenol was noted. Therefore, the water extract contained no bromide or iodide, and the brown liquid distillate was chromyl chloride. To confirm this statement, the following tests for chromyl chloride were performed:

To about 5 ml of the distillate was added a pellet of potassium hydroxide. The solution was acidified with nitric acid, some ether and a little hydrogen peroxide added, and the contents shaken. The upper ether layer was colored blue, which indicated the presence of chromium, showing that the brown distillate was chromyl chloride (Treadwell and Hall, '32), and that chloride was present originally in the water extract of the nerve fibers.

The same brown distillate was tested further with the diphenylcarbazide test (Feigl, '46). The diphenylcarbazide reagent was prepared by dissolving 0.5 gm syn-diphenylcarbazide in 70 ml 95% alcohol and then adding 25 ml glacial acetic acid and 5 ml distilled water (Glick, '49). The reagent was prepared immediately preceding use. A glass rod was dipped into a potassium hydroxide solution, then into some of the brown distillate, and finally into the diphenylcarbazide reagent on a spot plate. A deep violet color appeared instantaneously, indicating again that the brown distillate was chromyl chloride and that chloride was present in the original water extract.

These tests involving the formation of chromyl chloride are generally accepted as yielding positive results with chloride only, and serve to eliminate the presence of bromide and iodide when testing an unknown. The results of these tests are consistent with the assumption that the chloride comes from within the nerve fibers, though they do not prove this.

Extraction of Frommann's striations and identification of silver chloride. Although the water extract has been shown to contain chloride, it does not necessarily follow that the chloride is from the axons. A possibility still exists that the extracted chloride is from the interfibral space. As a further attempt to demonstrate that the chloride was extracted from the axons, silver chloride was produced in the axons and identified in the following manner:

Two stretches of sciatic nerve fibers, each about 4 cm in length, were spread on two slides and immersed in the silver nitrate-nitric acid reagent for 12 hours in the dark to allow the formation of silver chloride. One slide was exposed subsequently to strong sunlight to reveal the striations of Frommann in the axons. The other slide, without exposure to light, was washed with distilled water to remove excess silver nitrate-nitric acid reagent. The nerve fibers were lifted off the slide and placed into 5 ml of a warm ammonium carbonate-ammonia mixture for 40 minutes to extract the silver chloride. The ammonium carbonate-ammonia mixture was prepared by mixing 4 parts saturated ammonium carbonate solution with one part ammonia. The silver chloride in this extract was reprecipitated by acidifying with nitric acid. The precipitate was collected by centrifugation. After several washings with distilled water, the precipitate was treated with a 4% aqueous solution of potassium ferrocyanide for two minutes. It was again washed with distilled water. The supernatant fluid was decanted, and two drops of concentrated nitric acid were placed on the precipitate. An immediate, dramatic change of color occurred; the precipitate, previously white, became red-brown. This color change indicated that the silver chloride extracted from the nerve fibers was converted to silver ferrocyanide (white); the latter became silver ferricyanide (red-brown) upon treatment with nitric acid (Feigl, '46).

As a further control, the nerve fibers, which had been extracted with the ammonium carbonate-ammonia mixture, were exposed to strong sunlight. They failed to reveal any striation or any trace of silver precipitate. Thus, silver chloride was produced when nerve fibers were placed in the silver nitrate-nitric acid reagent in the dark. When exposed to strong sunlight, the silver chloride appeared as Frommann's striations in the axons. The silver chloride could be extracted with the ammonium carbonate-ammonia mixture and identified by converting it to silver ferricyanide. It is to be noted here that only silver chloride is completely soluble in the warm ammonium carbonate-ammonia mixture; silver bromide is very slightly soluble, while silver iodide is insoluble.

Again, these experiments are all consistent with the assumption that the silver chloride is formed within the nerve fibers, though they do not prove that it is present only there.

Identification and localization of silver chloride in nerve fibers. Since Frommann's striations are located in the axons, it may be inferred that chloride is present there. The preceding tests indicate that Frommann's striations may be a reaction product between the silver reagent and the chloride in nerve fibers. However, there is no conclusive evidence that Frommann's striations are composed of silver chloride. Therefore, if the striations are composed of silver chloride, it should be possible, by performing the above specific chemical test directly on nerve fibers, to convert the silver chloride to silver ferricyanide and produce striations of silver ferricyanide in the axons. Thus, if Frommann's striations could be rendered visible as silver ferricyanide, it would be permissible to conclude that the striations are composed of silver chloride since only silver chloride yields positive results when the above specific chemical test is applied to nerve fibers.

Nerve fibers were spread on a slide and immersed in the silver nitrate-nitric acid reagent for 12 hours in the dark without subsequent exposure to light. The fibers, in which silver chloride was formed, were lifted off and washed in distilled water to remove the excess silver reagent. The fibers were then put into a 4% aqueous solution of potassium ferrocyanide for 12 hours to convert the silver chloride to silver ferrocyanide, which was then changed to silver ferricyanide by transferring the nerve fibers to 50% nitric acid solution in distilled water. The nerve preparation turned yellow. The fibers were spread on a slide, a drop of glycerine and a coverslip were added. Microscope examination revealed yellow-brown striations in the axons (fig. 5). The striations had this light color, instead of the red-brown color of the silver ferricyanide in the previous test, probably because the nitric acid used was only a 50% solution. Concentrated nitric acid was purposely avoided because it was desired to prevent excessive damage to the fibers. On the basis of the fact that Frommann's striations are located in the axons and the observation that the striations may be converted to silver ferricyanide, it is concluded that Frommann's striations are composed of silver chloride and that silver chloride is formed in the axons where the chloride is present originally.

Production of Frommann's striations after extraction. Since Frommann's striations are composed of silver chloride, it would be interesting to see if, after extraction, Frommann's striations could be produced by restoring chloride.

Nerve fibers were teased on a slide and immersed in distilled water for 40 minutes to extract chloride. To restore chloride, the fibers were then covered with a 0.9% aqueous solution of sodium chloride for 10–12 minutes. The specimen was rinsed in distilled water for 30 seconds and then immersed in the silver nitrate-nitric acid reagent in the dark. After one to one and a half hours, the slide was exposed to strong sunlight. Striations were revealed in the axons (figs. 6 and 7). A control slide treated simultaneously in the same manner, but without restoration of chloride, revealed no Frommann's striations or any silver precipitate whatsoever.

This test indicates that the Frommann's striations are a result of reaction between silver and chloride, and the latter can be extracted from and can be restored to the axons.

DISCUSSION

Lorente de Nó ('50) believes that the connective tissue sheath of nerve may be ignored as a significant barrier to diffusion of solutes or in the analysis of potentials. On the other hand, the connective tissue sheath has been held to be an effective barrier to diffusion of solutes into nerve (Feng and Liu, '49) and to influence the distribution of current in nerve (Rashbass and Rushton, '49). It was with these points in mind that, throughout this study, the connective tissue sheath was removed, and the nerve fibers were spread on a slide before tests were performed on them. It was felt that nerves thus treated would allow the reagents to act more directly on the individual fibers.

The striations of Frommann are formed when the silver nitrate reagent enters the nerve fiber at the node, diffuses into the axon, and reacts with the chloride present in the axon to produce a striated precipitate of silver chloride. Thus, the striations of Frommann may serve to indicate the presence and localization of chloride in axons. It is not intended here to state that the chloride of the axon is distributed in a striated arrangement or to deny that chloride is present in the intercellular spaces between the nerve fibers. However, it is desired to stress that the present observations indicate that chloride is present also within the axons.

None of the tests used in this study, when considered alone, is sufficiently specific for the identification of chloride or for the determination of its exact location. Indeed, to date, there is no single ideal chemical test for chloride, especially when applied to biological material. In this study, it has been shown by a number of tests that the water extract of nerve contains chloride; that the chloride reacts with the silver nitrate reagent to form silver chloride; that by specific chemical tests and direct microscopic observation silver chloride is located in the axons; and that the chloride, after extraction, can be restored to the axons to form the striations of Frommann. Only by considering all these facts may it be concluded that chloride is present within the axons.

There is, of course, the possibility that chloride from the interfibral space of the nerve may enter the axons during treatment with the silver nitrate-nitric acid reagent to produce Frommann's striations. However, in the teased preparations used in this study, intercellular chloride is readily accessible to the silver reagent. The silver from the reagent and the chloride from the interfibral space would form immediately a water-insoluble precipitate of silver chloride outside of the axons. It is unlikely that this water-insoluble silver chloride could enter the axons and form there a striated precipitate. There is every indication that the chloride made visible by the formation of Frommann's striations was already present in the axons.

The presence of chloride in axons, in muscle fibers (Wilde, '45; Steinbach, '47) and, indeed, in many other cells (Manery and Hastings, '39; see Heilbrunn and Hamilton, '42, for discussion and references) negates the well established view that chloride occurs only extracellularly.

CONCLUSIONS

Histochemical tests and analyses of extracts of nerve fibers indicate that:

1. The striations of Frommann are composed of silver chloride.
2. Chloride is present in mammalian axons.
3. Frommann's striations serve as an indicator of the presence and localization of chloride in axons.

ACKNOWLEDGMENTS

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PLATE 1

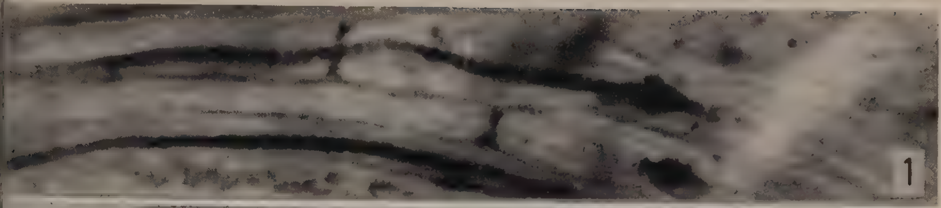
EXPLANATION OF FIGURES

All figures are of teased fibers from the sciatic nerves of white rats. All photomicrographs are of the same magnification: × 1000.

1–4 Striations of Frommann in axons of nerve fibers treated with the silver nitrate-nitric acid reagent in the dark with subsequent exposure to strong sunlight. Note, in figure 4, the node of Ranvier where the reagent first enters the axon.

5 Axon showing striations of silver ferricyanide, which was converted from silver chloride by treatment with potassium ferrocyanide and nitric acid.

6 and 7 Striations in extracted nerves in which chloride has been restored.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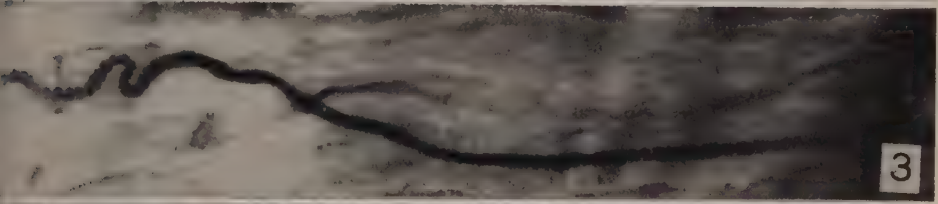
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PRELIMINARY STUDIES ON OXYGEN CONSUMPTION OF LUMINOUS BACTERIA MADE WITH THE OXYGEN ELECTRODE

M. JAY GOODKIND AND E. NEWTON HARVEY
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SIX FIGURES

INTRODUCTION

When a suspension of luminous bacteria is allowed to stand, the light remains of nearly constant intensity for a certain time, then begins to dim rapidly and in 20 to 30 seconds has practically disappeared, except at the surface in contact with air. The respiring bacteria have used all the dissolved oxygen. If air is again bubbled through the suspension, light reappears and is brighter than before (the flash), but quickly returns to the same intensity or to a lower intensity than that of the original suspension in air. The course of light intensity change, including the "flash," has been recorded and studied by Harvey ('32), by Johnson, van Schouwenburg and van der Burg ('39) and by Schoepfle ('40), but the accompanying changes in oxygen concentration of the medium have never been directly measured.

The time for the suspension to dim ("dimming time") is proportional to the amount of dissolved oxygen and inversely proportional to the number of bacteria (Harvey, '28), and has been used as a rough measure of the rate of respiration of the bacteria. For this to be true the respiration of the bacteria should remain constant until a relatively low oxygen pressure is reached and a plot of oxygen concentration in the suspension versus time should be a straight line.

Shoup ('29) found that oxygen consumption of a marine luminous bacterium remained constant from 21% oxygen to about 2.5% oxygen and that the respiration was reduced only 10% at 1.2% oxygen. The purpose of the present paper is to verify this relation by recording the decrease in dissolved oxygen in the medium and to study some of the variations encountered with different strains of bacteria and with the same strain at different culture ages or suspended in different media. In addition to oxygen concentration, the luminescence intensity of the suspension has been measured simultaneously.

While it is a simple matter to measure continuously the light intensity of luminous bacteria, a continuous measure of dissolved oxygen presents greater difficulties. The oxygen electrode appears suitable for this purpose, providing its limitations and peculiarities are taken into account. The experience of the authors with this electrode, applied to a suspension of luminous bacteria, has been on the whole favorable. It would be ideal if a record of oxygen pressure could be obtained during the period when the light intensity of the bacteria is rapidly decreasing. Such a measurement would involve working in a region where the oxygen pressure is less than 0.25% (Shoup, '29) to 0.14% (Shapiro, '34),[1] a refinement which demands greater sensitivity than that used in the present study, together with automatic recording of small currents during a time interval of a few seconds.

METHOD

Application to cells and tissues of the polarographic method of Heyrovsky for measuring oxygen has been made by Baumberger ('39) and Winzler ('41), using the dropping mercury electrode, and by Blinks and Skow ('38), Davies and Brink ('42), Haxo and Blinks ('50) and others, using the platinum electrode. Davies and Brink have developed Pt microelectrodes (25 μ diam.) of the open and recessed type for study of local oxygen tensions near capillaries in the brain.

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[1] Unpublished experiments of J. W. Hastings in the senior author's laboratory indicate that the curve relating luminescence intensity and oxygen concentration is a rectangular hyperbola approaching the 100% intensity value asymptotically.

As it was feared that mercury might injure the bacteria, a platinum electrode was selected for the work. Those familiar with the polarographic method applied to determination of oxygen with a platinum electrode will recognize the two difficulties which must be guarded against: (1) Spontaneous changes in the electrode requiring frequent recalibration; (2) Utilization of oxygen by the electrode, thus establishing a diffusion gradient which necessitates calibration in exactly the same manner as that used in taking the experimental readings. Since the suspension of luminous bacteria may be of large volume and there are no local changes in oxygen tension in the suspension, the electrode can be large. However, it was found that large pieces of platinum foil gave poor ''plateaus'' and that the readings were not reproducible, possibly because the electrode surfaces were uneven, giving interference patterns in the diffusion fields. After considerable experimentation, a rather small electrode of the open type was adopted and has been used throughout the experiments. This electrode gave a good plateau (fig. 1) and yet allowed sufficient current to flow to be easily measured by the galvanometer without amplification.

The electrode was made of a 0.4 mm diam. Pt wire sealed in glass, ground to the surface of the glass and then polished. In constructing the electrode a drop of molten lead glass was placed on the end of the Pt wire and the wire sealed with an oxygen-gas flame in the end of a length of 4 mm bore soda glass tubing, taking care to avoid trapping air bubbles around the wire. Grinding and polishing were then continued until the Pt and glass surfaces were smooth, as shown in figure 1 (insert). A typical current-voltage curve for this electrode is also reproduced in figure 1.

The bacterial suspension (75 cm^3) was placed in a test tube with a rubber stopper through which passed the glass tube containing the Pt electrode, a salt bridge from the calomel electrode and an inlet and outlet tube for gases. Connections from the Pt and calomel electrode were made to the potential divider through a Leeds and Northrop Series R galvanometer with a sensitivity of 0.0028 microamperes per mm and a

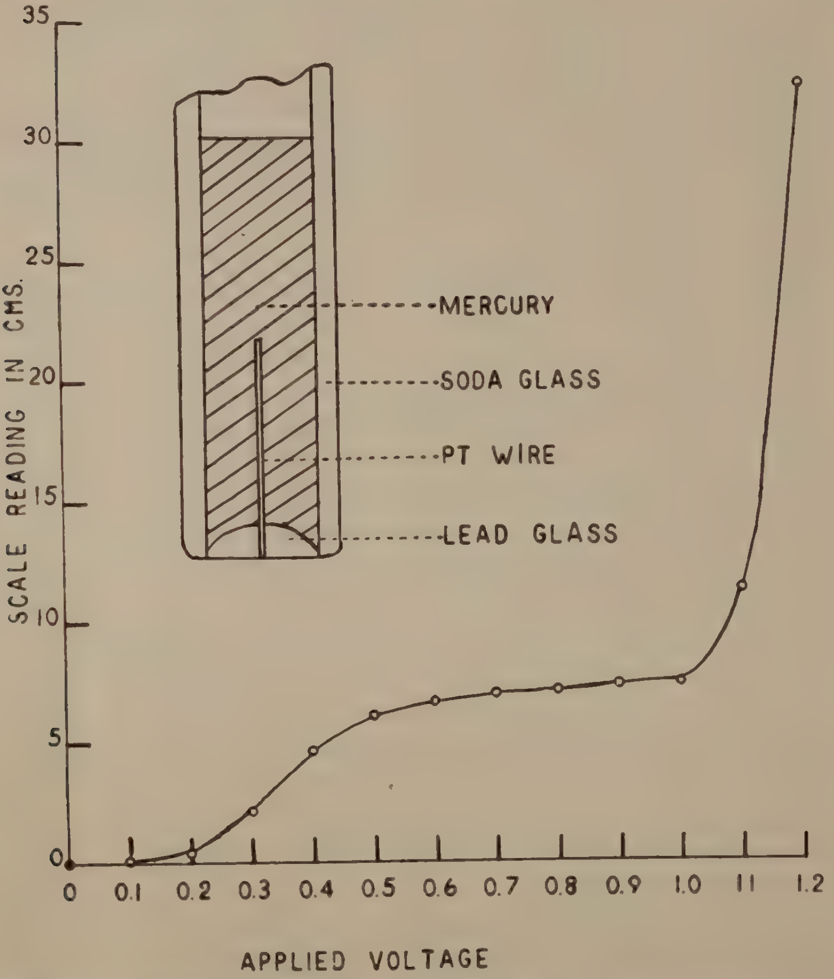

Fig. 1 A typical current-voltage curve for the open platinum electrode, shown in longitudinal section in the insert.

period 3 seconds. In all graphs the current is measured as a scale reading. A Weston voltmeter measured the voltage from the voltage divider of 429 ohms, across a 1.5 volt dry cell. For all measurements 0.6 volt was applied to the electrode system.

Since bivalent cations like Ca and Mg have some effect on the oxygen electrode, the calomel electrode was made up with Hg-Hg_2Cl_2-3% NaCl, the salt bridge was 3% NaCl and the bacteria were suspended in 3% NaCl. In some later experiments, to prevent changes in pH, the bacteria were suspended in "PN," a mixture of equal parts of 0.5 m NaCl (about 3%) and 0.25 m phosphate buffer of pH = 7.2. The salt bridge was also filled with PN. Both media gave essentially similar results.

Two strains of bacteria were used — Achromobacter fischeri and an unidentified Bermuda species known as No. 257,[2] probably a strain of Achromobacter fischeri. They were grown in Petri dishes on 1.5% agar containing 3% NaCl, 1% glycerol, 2% peptone and $CaCO_3$ to excess, for 12 to 36 hours at room temperature, about 20°C. To prepare suspensions the bacteria were brushed off the agar into 3% NaCl or PN solution.

Calibration of the electrode was made with various mixtures of air (21% oxygen) stored under pressure in a tank and tank nitrogen, assumed to contain 1% of oxygen. The mixtures were made by adjusting the rates of gas flow by a valve and allowing the gases to displace water in an inverted graduated cylinder. By means of a three way cock it was possible to measure the time for 100 cm^3 of water to be displaced (1) by air, (2) by nitrogen and (3) by the mixture of the air and nitrogen whose proportions could be calculated. The various mixtures were bubbled through the electrode vessel containing 3% NaCl or PN for 30 min. to saturate at a known oxygen concentration. The flow of gas was then stopped, 0.6 volts applied to the electrode, and readings of current made every 15 seconds for 5 minutes. The curves shown in figure 2 (left) were obtained.

If the electrode is a satisfactory one, there should be linearity between oxygen tension and current and the straight line should pass through zero of *each* axis. Figure 2 (right) shows that the electrode used in the experiments possessed the proper linearity. To construct this curve the oxygen tensions were expressed in per cent oxygen and the current was taken at a time when it had reached a practically steady value, 2.5 minutes. It is not known why the point at 4.68% O_2 deviates.

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[2] Kindly supplied by Dr. E. R. Baylor.

The experimental runs with the bacterial suspensions were carried out in a similar way. The suspension of bacteria was shaken with air, placed in the vessel, bubbled with air for 30 minutes and 0.6 volt applied to the electrode just before bubbling was stopped. Readings were then made every 15 seconds.

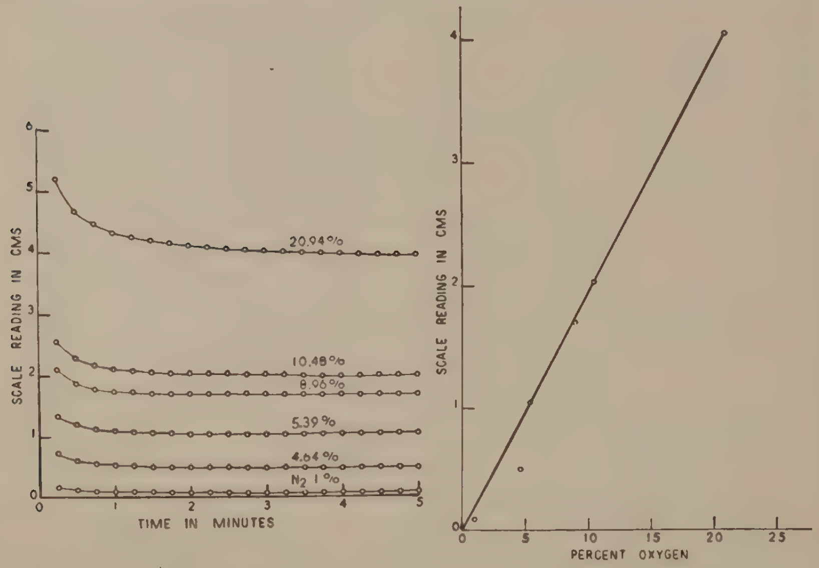

Fig. 2 Left. Curves of current (in centimeters) with 0.6 volt applied potential versus time for various percentages of oxygen used in calibrating the electrode.

Right. Current after 2.5 minutes with 0.6 volt applied potential as a function of oxygen concentration in 3% NaCl.

After each bacterial experiment, the electrode was calibrated for constancy by taking a series of readings at 15 second intervals in air saturated (21% oxygen) 3% NaCl solution.

At the same time that the oxygen concentration readings were made, the light intensity of the bacteria was measured by means of a photomultiplier tube (R.C.A. 931 A) placed near a window in the bacterial vessel. The photomultiplier tube had a suitable power supply and current meter built by the Audiotone Oscillator Co., Bridgeport, Conn. The complete setup was enclosed in a dark box with curtains so that experiments could be made in a dimly lighted room. Experiments were carried out at room temperature, which varied from 18 to 21.3°C. on different days.

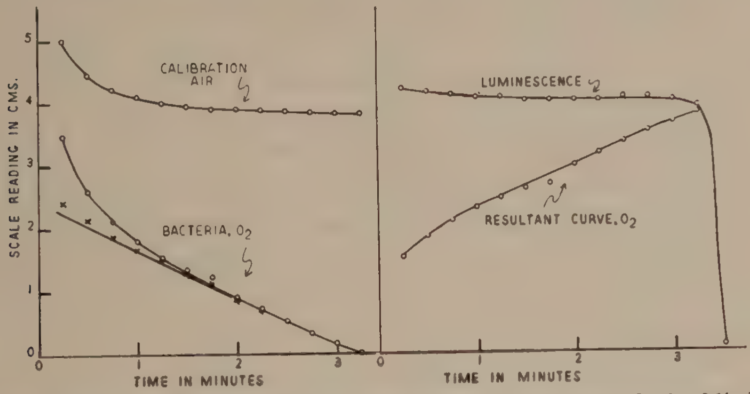

Fig. 3 Left. The upper curve is a calibration of the electrode in 3% NaCl saturated with air, giving readings of current as a function of time after 0.6 volt has been applied. The lower curve shows the change of current with time as a suspension of Achromobacter fischeri (13 hour culture) uses the oxygen in the 3% NaCl. The line of crosses is a corrected curve for oxygen concentration versus time.

Right. The upper curve shows the change of light intensity of the same bacterial suspension with time and the lower curve gives the total oxygen consumed, as explained in the text. In this and all subsequent figures the scale reading in centimeters refers to current when 0.6 volt is applied to the oxygen electrode, while the scale reading in units refers to luminescence intensity.

RESULTS

Typical curves, relating decrease in light intensity and oxygen concentration with time, for a suspension of Achromobacter fischeri in 3% NaCl (a 13 hour culture) are shown in figure 3. In figure 3 (left), the current in terms of scale

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readings is plotted versus the time the thoroughly aerated bacterial suspension was allowed to stand undisturbed (lower curve) and the change in current of the same electrode when it stands in air saturated 3% NaCl solution (upper curve). It will be observed that about three minutes are necessary for the electrode to come to equilibrium. Since the electrode gives a higher value for current when readings are first made, the lower curve of oxygen concentration of the bacterial suspension versus time must be corrected at each time interval by subtracting the per cent excess reading obtained from the upper calibration curve in air. When this is done, the oxygen consumption is found to be nearly linear, as shown by crosses.

In figure 3 (right lower curve) the total oxygen consumed, obtained by subtracting the current readings on the bacterial curve from the current readings in calibrating the electrode, has also been plotted. It gives a straight line except for deviations at the beginning and the end. In figure 3 (right upper curve) the luminescence intensity of this same bacterial suspension has been plotted. It will be observed that the light intensity remains nearly constant until the oxygen concentration has been reduced to nearly zero, when rapid dimming begins.

Since the bacteria are suspended in 3% NaCl containing only the small amount of nutrient which may have been washed from the culture medium, it is to be expected that the light intensity will decrease with time, even though the oxygen concentration is ample to maintain the luminescence at its maximum. An example of this behavior is seen in figure 4, in which a 36 hour culture of A. fischeri and a 12 hour culture of the Bermuda strain are compared for change in luminescence and change in oxygen concentration. The decrease in light intensity with time is especially marked in the case of the Bermuda strain. This decrease in light intensity is quite independent of oxygen concentration and occurs in suspensions in 3% NaCl through which air is continually bubbled.

If the Bermuda strain is allowed to stand under anaerobic conditions for even a few minutes, the flash of light on readmitting oxygen is reduced and the subsequent light intensity considerably less than before anaerobiosis, with some recovery of lumniscence intensity as time goes on. The decreased luminescence intensity has been attributed to destruction of luciferase by proteolytic hydrolysis (Johnson, van Schouwenburg and van der Burg, '39), a process which occurs very quickly in some luminous bacteria in absence of oxygen, less quickly in others. The proteolysis ceases in air and luciferase is reconstituted, provided the bacteria have not been kept too long under anaerobic conditions.

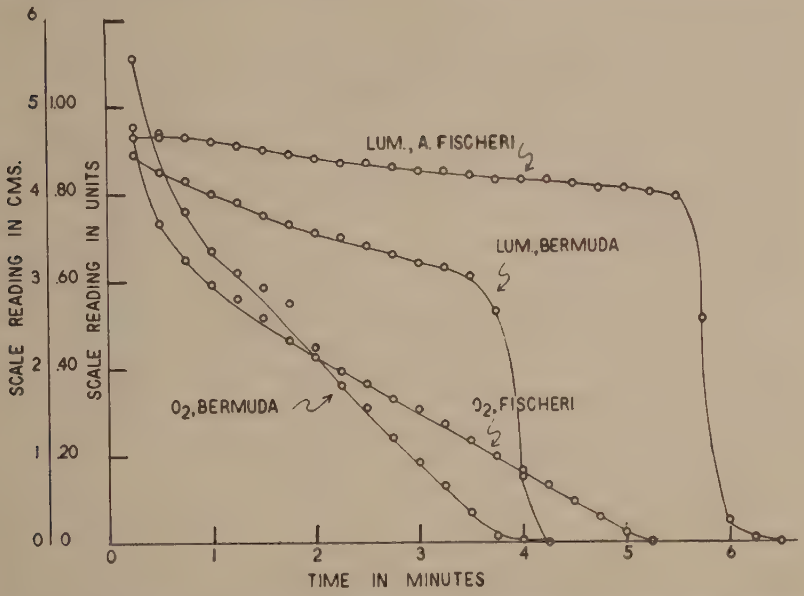

Fig. 4 Luminescence intensity and oxygen concentration curves for Achromobacter fischeri (36 hour culture) and the Bermuda strain (12 hour culture). Coordinates as in figure 3.

Since the 3% NaCl suspension medium is unbuffered, the acidity continually increases, due to accumulation of CO_2 and sometimes organic acids, frequently becoming as acid as $pH = 5.5$. A buffered suspension medium was therefore tried, a mixture of equal parts of 0.5 m NaCl and 0.25 phosphate buffer at pH = 7.2, known as PN. The electrode calibration does not remain as constant in this medium. However, it was found that the behavior of the bacterial suspensions was practically the same in PN as in 3% NaCl solution, indicating that the decrease in light intensity with time is due to decrease in nutrient rather than accumulation of acid.

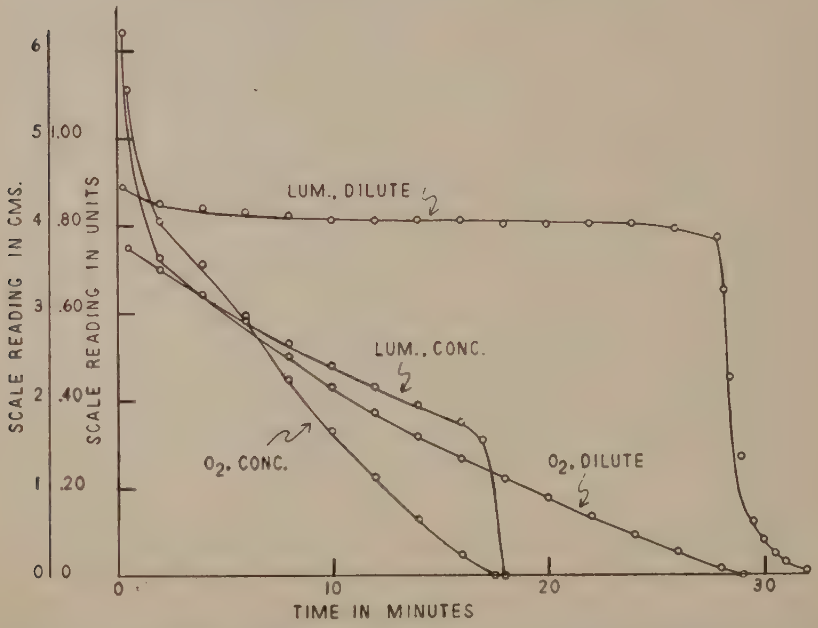

Fig. 5 Luminescence intensity and oxygen concentration curves for the Bermuda strain in a dilute and more concentrated suspension. Coordinates as in figure 3.

In all, some 37 different suspensions of luminous bacteria have been studied in various dilutions and from cultures of different ages. Sample results are shown in figures 5 and 6. The same general behavior is to be noted but the measurements on the same organisms with the same culture age, carried out from day to day have not always been consistent. Usually the uncorrected oxygen concentration curve for a dilute suspension of the Bermuda strain is slightly concave to the time axis, but occasionally the decrease in oxygn concentration is linear over a wide range. Various differences will be noted from inspection of the figures. These are probably to be related to the previous history of the bacteria suspension, but the data at hand are insufficient for analysis at this time.

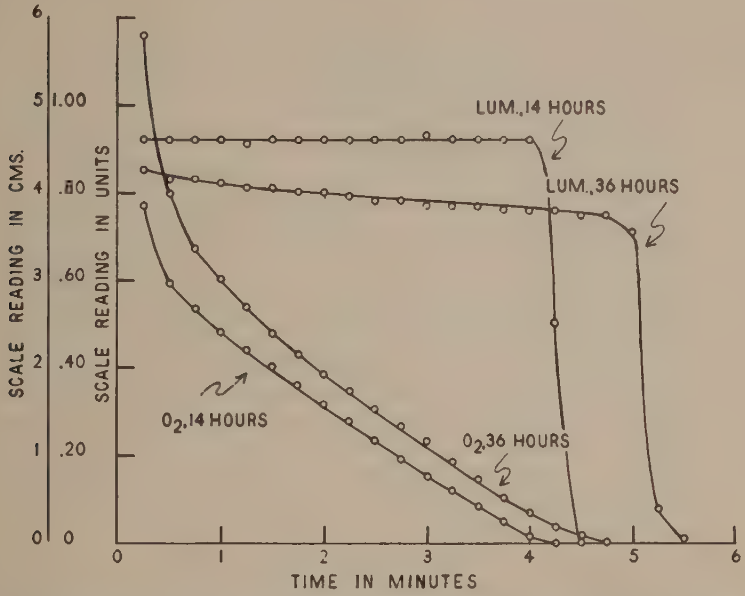

Fig. 6 Luminescence intensity and oxygen concentration for suspensions of A. fischeri of two different culture ages. Coordinates as in figure 3.

SUMMARY

The platinum oxygen electrode has been used to measure the oxygen concentration in suspensions of two species of luminous bacteria as they use the dissolved oxygen of the medium in respiratory processes. The decrease in oxygen concentration is usually linear, indicating a constant respiratory rate until the concentration approaches 1%. Luminescence intensity of the suspension was followed by photocell measurements at the same time as the change in oxygen concentration. The light intensity begins to change rapidly at

less than 1% oxygen. From these preliminary results it appears possible to follow the rather rapid utilization of oxygen in concentrations below 1% oxygen by using the platinum electrode with a more sensitive current measuring device and automatic recording. We gratefully acknowledge the interest and helpful advice of Dr. Frank Brink, Jr., during the course of this investigation.

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PHOTORECOVERY FROM THE EFFECTS OF ULTRAVIOLET RADIATION IN SALAMANDER LARVAE[1]

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FIVE FIGURES

Since photorecovery after ultraviolet radiation was described two years ago by Kelner ('49) in a fungus it has been found elsewhere in the animal and plant kingdom. The present paper reports it in salamander larvae; apparently the first example found in a vertebrate, or any animal above the echinoderms. The characterization of this effect and its differentiation from other photobiological phenomena with which it may be confused are also dealt with.

EXPERIMENTAL

Larvae of *Amblystoma maculatum* were used in most of the experiments; larvae of *A. opacum* in a few, with similar results. The animals were raised in the laboratory from eggs collected in North Carolina or the Princeton area. They were kept in individual dishes of pond water and fed a mixed diet of *Enchytrea* worms and *Daphnia*.

For exposure to ultraviolet radiation the larvae were placed in flat dishes with just sufficient water to cover them. The source of radiation was an intermediate pressure mercury arc, which was monitored with a photoelectric cell. The characterization of the radiation and the method of measurement of dosage is described elsewhere (Blum, Kirby-Smith and Grady, '41). The intensity incident on the animals was approximately 3×10^4 ergs per cm^2 per second of radiant energy of wavelengths 0.313 μ and shorter. The dose was adjusted for each experiment. In most instances the exposure lasted less than a minute, never longer than a few minutes.

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[1] Briefly reported by the authors (1950).

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[3] National Institutes of Health, Public Health Service, Federal Security Agency.

Illumination with "visible" radiation was from "fluorescent" lamps placed approximately 10 cm above the dishes containing the animals. Two types of lamp with different spectral ranges were used. The emission spectra of these lamps are shown in figure 3, at A and B. Source A was a bank of two 20 W calcium-ceasium fluorescent lamps[4] the spectrum of which has its maximum at 0.36 μ and its limits approximately 0.32 μ to 0.46 μ. Source B was a bank of two 15 W "daylight" lamps which have a spectral range from approximately 0.4 μ to 0.7 μ. In some experiments restricted wavelengths of illuminating light were obtained by placing glass color filters over the dishes containing the animals, the walls of the dishes being wrapped with aluminum foil to eliminate stray light. Transmissions of these filters are also shown in figure 3. Although the total energy output of these lamps is not high, the dishes were kept in running tap water during illumination to guard against undue heating.

Single doses of ultraviolet radiation. In preliminary experiments the approximate lethal dose was determined for larvae of various ages. The lethal dose varies with the age of the animal. For example larvae of Harrison stage 28[5] were killed by a dose of approximately 2×10^6 ergs per cm^2 whereas older larvae, 37 mm long, required approximately 3×10^7 ergs per cm^2 to kill.

Attempts to demonstrate photorecovery after single lethal doses were unsuccessful. Identically treated animals placed in dark and in light died at the same time.

[4] These lamps were supplied by courtesy of the Westinghouse Electric Corporation.

[5] Drawings representing the stages of development of the Amblystoma larva as numbered by R. G. Harrison appear in Hamburger ('42), pp. 202–204.

Repeated doses. Photorecovery was easily demonstrable, however, when the animals were subjected to repeated doses. The dose was adjusted according to age and was usually a small fraction of that required to kill if given singly. In most cases the exposures to ultraviolet radiation were made 5 days per week (Saturday and Sunday being omitted). Figure 1 illustrates a typical experiment of this type, in which three

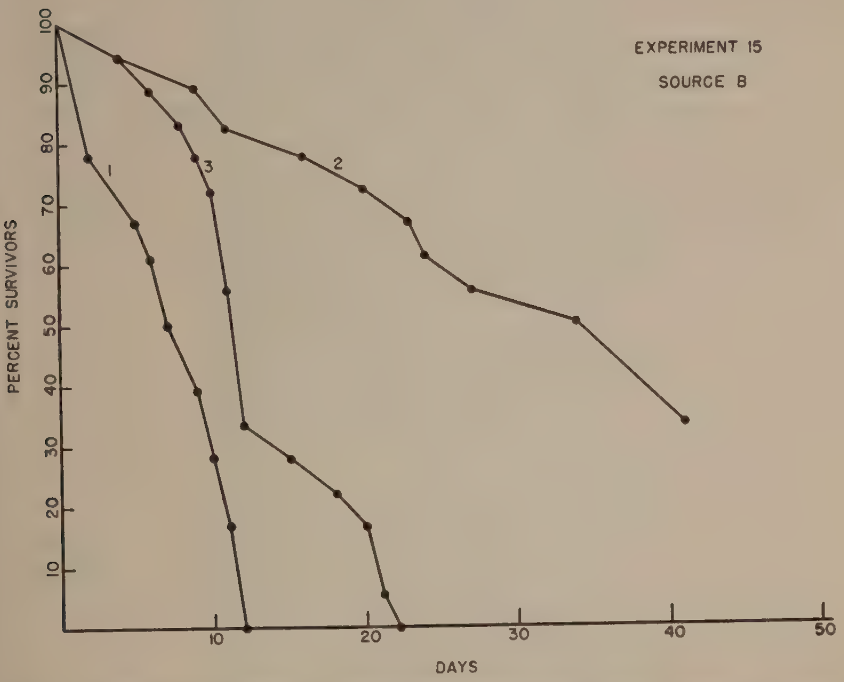

Fig. 1 Survival of larvae of *Amblystoma maculatum* which received repeated doses of ultraviolet radiation. Each dose, which was given 5 days per week, was 8 × 10^5 ergs per cm^2 of mercury arc radiation of wavelengths 0.313 μ and shorter delivered in about 30 seconds. The larvae were 22 to 24 mm long. The dose was approximately 7% of a single lethal dose.

Curve 1. A group of 18 animals kept in darkness between exposures to ultraviolet radiation.

Curve 2. A group of 18 animals illuminated with source B for 4 hours *after* each dose of ultraviolet radiation.

Curve 3. A group of 18 animals illuminated with source B for 4 hours *before* each dose of ultraviolet radiation.

groups of larvae were subjected to repeated doses of ultraviolet radiation, but to different conditions of illumination during the intervening periods. Curve 1 represents survival of a group of animals kept in darkness between exposures to ultraviolet radiation. The last animal of this group died in 12 days. The group represented by curve 2 received 4 hours of illumination with source B immediately following each exposure to ultraviolet radiation. A considerable portion of the animals in this group still survived after 40 days. Nine experiments of this general type, involving a total of 478 animals were carried out with different doses, animals of different ages and different conditions of illumination. In all cases those illuminated survived longer than those in the dark.

We are not particularly concerned here with the cause of death of the animals since survival is used only as a crude index of the extent of injury by ultraviolet radiation. Some of the gross changes observed in the larvae exposed to ultraviolet radiation are, however, of interest. Such animals failed to grow as rapidly as normal controls. The gills were reduced in size relative to the rest of the body. There was sloughing off of the epidermis varying in degree with the dose of radiation Regeneration of amputated limbs was slower than in controls. All these effects were less obvious in animals kept in the light than in those kept in darkness. Figure 2 includes photographs of larvae subjected for three months to very low doses of ultraviolet radiation. These illustrate the effect of this radiation and the influence of subsequent illumination. Even without a more thorough analysis of these changes it seems reasonable to say that illumination markedly increases the ability of *Amblystoma* larvae to recover from the effects of the ultraviolet radiation. We may, then, draw a comparison between these findings and photorecovery or photoreactivation in other systems.

Illumination of normal animals. Characteristically the light that brings about photorecovery after ultraviolet radiation is without effect on the normal organism. This was checked

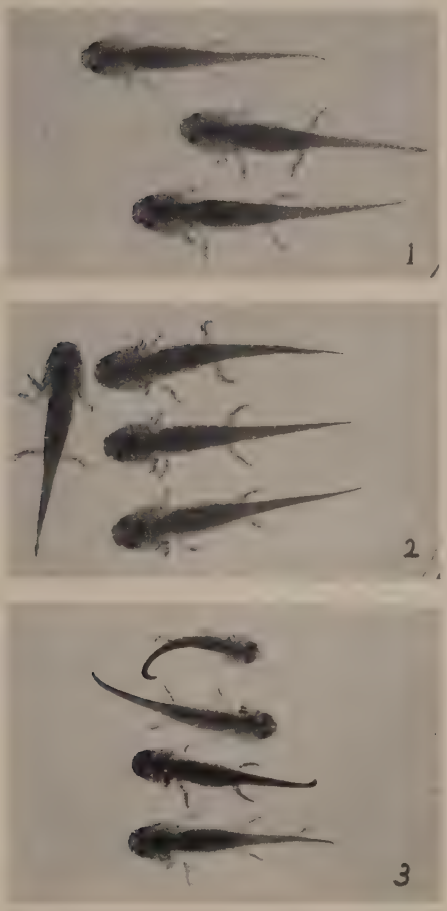

Fig. 2 Larvae of *Amblystoma opacum.* The larvae were 32 to 36 mm long at the beginning of the experiment. All had the right gills removed and the right forelimb amputated through the humerus three months before the photographs were taken. The animals in 1 received no ultraviolet radiation. The animals in 2 and 3 were exposed to ultraviolet radiation 5 days per week during a period of three months. Each dose was 1.6×10^4 ergs per cm^2 of mercury arc radiation of wavelengths $0.313\,\mu$ and shorter, delivered in about 6 seconds, which was approximately 2% of a single lethal dose. The first dose was given shortly after the amputation of the gills and limbs. The animals in 2 were illuminated with source B for 5 hours after each dose of ultraviolet. Two of the animals in 1 received the same illumination each day, the other remained in darkness; all show complete regeneration of limbs and gills. The animals in 3 remained in darkness between the exposures to ultraviolet radiation.

in the present case by placing groups of normal *Amblystoma* in darkness and in light. The larvae survived as well under the one condition as the other, and showed no difference in growth or other behavior. Regeneration of amputated limbs and gills went on as rapidly in darkness as in light (see fig. 2).

Illumination before ultraviolet radiation. It is characteristic of photorecovery that illumination prior to dosage with ultraviolet radiation does not affect subsequent recovery. Experiments were set up to test this point, one of which is illustrated in figure 1 where group 3 received the same amount of illumination as group 2, but in the former this was given on each day just prior to the dosage of ultraviolet radiation instead of just following as in the latter. It is seen that the animals in group 3 survived better than did the animals in the dark (group 1) but not so well as those that received their illumination *after* exposure to ultraviolet radiation (group 2). In this particular experiment the animals of all groups were under the illuminating lamps at the same time. The dish containing the dark sample (1) was covered with aluminum foil, sample 3 received its dose of ultraviolet radiation at the end of the 4-hour illumination period, sample 2 just before illumination. All the animals were kept in the dark room except during the time required for exposure to ultraviolet radiation and the illumination period. Thus, except for the illumination factor, all the animals received the same treatment.

Comparison of the curves in figure 1 may on first notice suggest that ''preillumination'' enhances the recovery of the larvae after exposure to ultraviolet radiation. This is not the conclusion to be reached, however, since the illumination prior to exposure to ultraviolet is really (except for the first exposure) an illumination beginning 20 hours after the previous dose of ultraviolet radiation. What the curves indicate is that photorecovery is still possible 20 hours after exposure to ultraviolet radiation, although it is less in amount than the photorecovery immediately after irradiation.

We may compare these results with those on the egg of the sea urchin, *Arbacia punctulata,* where recovery falls off as the normal rhythm of cell division is approached. This is clearly shown in text-figure 1 of the paper by Blum, Loos and Robinson ('50). Recovery is complete in that case in about 100 minutes, that is, at the end of that time the rate of cell division has returned to normal. It the eggs remain in the dark during this period, they show recovery but at a much slower rate, so that at the end of 100 minutes the intercleavage interval is still longer than the normal. Kelner ('49b) found also that the degree of photoreactivation of *Escherichia coli* falls off with time after exposure to ultraviolet radiation. We assume similar happenings in the case of the *Amblystoma* larva. During 20 hours of darkness after ultraviolet radiation some recovery takes place but much more slowly than it would in light. At the end of this time illumination enhances recovery but not so much as if applied earlier. So larvae illuminated 20 hours after exposure to ultraviolet do not survive as well as larvae illuminated immediately after.

If our interpretation is correct, increasing the interval between exposure to ultraviolet and illumination with visible should decrease survival. An experiment set up to examine this point compared the effect of illumination two days after each exposure to ultraviolet with illumination immediately after. The experiment was not carried to the death of the animals, but at its termination the group illuminated two days after ultraviolet showed damage comparable to that of the group kept in the dark, whereas the group illuminated immediately after ultraviolet appeared virtually normal. The experiments may be interpreted, then, as showing that pre-illumination does not influence recovery, and also that the recovery process is much slower in the case of the salamander larva than it is in bacteria, in fungi, or in the sea urchin's egg.

Spectral range of radiation causing photorecovery. Another characteristic of the photorecovery process is that, insofar as studied, the same general range of wavelengths is involved no matter what the organism concerned. In bacteria, in fungi, and in the *Arbacia* egg the limits lie, roughly in round numbers, between 0.3 μ and 0.5 μ. Dulbecco's ('50) action spectrum for reactivation of bacteriophage adsorbed on *E. coli* indicates a maximum in the neighborhood of wavelength 0.37 μ. Kelner ('51) reports an action spectrum for *E. coli* that agrees well with that of Dulbecco, but one for *S. griseus* that is similar in shape but has its maximum at about 0.42 μ. Although the sea urchin egg does not offer a suitable material for the determining of an action spectrum with any degree of detail, it is significant that the wavelength range for photoreactivation is approximately the same in the *Arbacia* egg as in bacteria and fungi. It is reasonable to conclude that similar chemical compounds are the light absorbers concerned in the primary photochemical process in all these cases. The following experiments indicate the same approximate spectral range for photorecovery in *Amblystoma* larvae.

Larvae subjected to repeated doses of ultraviolet radiation were illuminated with restricted wavelength ranges for a given period of time after each dose. The spectral emissions of the two sources (A and B) and the spectral transmissions of the filters used to limit the wavelengths are indicated in figure 3. Ten animals were illuminated under each filter. The results of three of the experiments are shown in figures 4, 5, and 6. If the survival times indicated there are compared in terms of the corresponding spectral ranges of illuminating radiation indicated in figure 3 consistency is evident. Figures 4 and 5 represent experiments using source B with filters cutting off various portions of the longer wavelengths from this source. Under filters which cut off wavelengths shorter than approximately 0.5 μ (e.g., 3384, 3484, and 2424) larvae survived only about as long as in darkness. They survived much longer under filters which pass most of the wavelengths shorter than 0.5 μ (e.g., 3389, 7380, W. G. and 3387). Filter 3385, which allows only a small part of the radiation shorter than 0.5 μ to pass through increased survival only slightly. Source A, which delivers princially wavelengths in the very near ultraviolet was used in the experiment described in figure 6. Filters which pass most of this radiation permitted good survival of the animals. Two additional experiments yielded results consistent with the three described in the figures. These experiments permit us to place the long wavelength limit for photorecovery in the salamander larvae at roughly 0.5 μ. The short wavelength limit may be placed with somewhat less certainty at about 0.3 μ.[6] If the studies are compared with those of Blum, Loos and Robinson ('50) on the *Arbacia* egg it is clear that approximately the same spectral limits apply. It seems safe to say that in the cases studied, in *Streptomyces griseus,* in *E. coli,* in the *Arbacia*

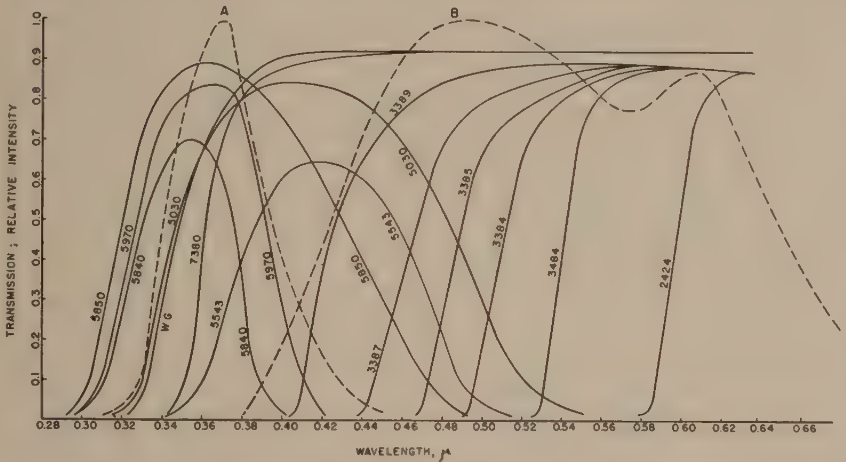

Fig. 3 Spectral emission of "fluorescent" lamps used as sources of "illumination."

A. Spectrum of a calcium-caesium lamp.

B. Spectrum of a "daylight" lamp.

Spectral transmission of filters used to limit wavelength ranges. W. G. is window glass. The numbers accompanying the other curves are the code numbers for Corning glass filters.

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[6] This material does not lend itself to the determination of a detailed action spectrum (see e.g. Blum, '50) so more exact optical and radiometric measurements would not seen justified. Dulbecco ('50) has shown in the case of *E. coli* a pronounced intensity dependence at high intensities and this would add to the difficulty of interpreting such measurements with the present material.

egg and in the salamander larva, the spectral ranges are approximately the same. This alone suggests that we deal with essentially the same mechanism.

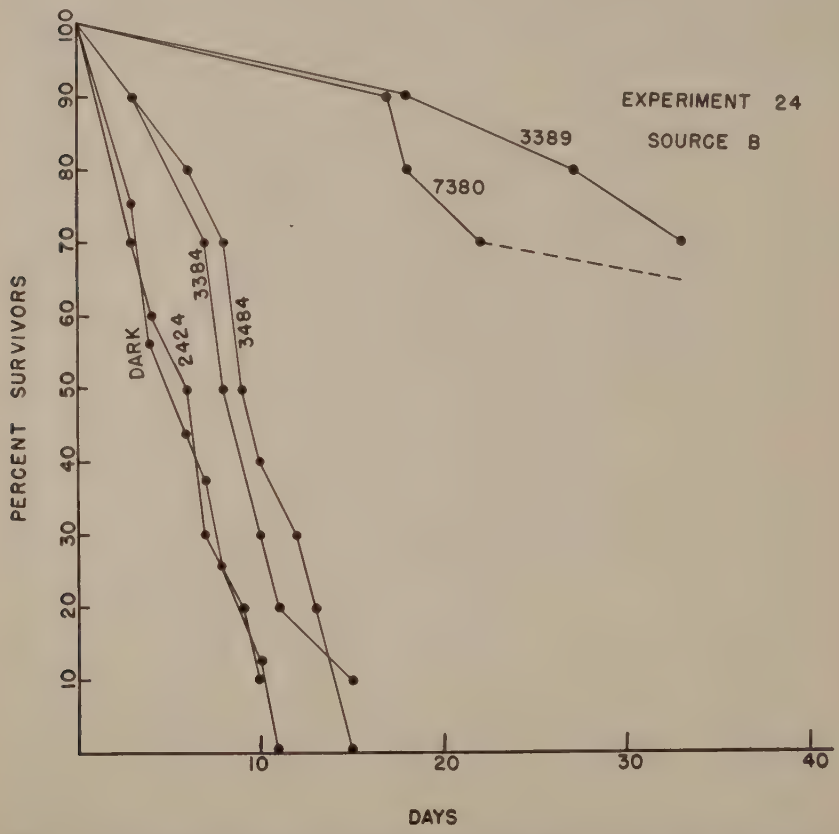

Fig. 4 Survival of larvae of *Amblystoma maculatum* which received repeated doses of ultraviolet radiation. Each dose, which was given 5 days per week, was 6.4×10^5 ergs per cm^2 of mercury arc radiation of wavelengths $0.313\,\mu$ and shorter, delivered in about 24 seconds. The larvae were about 13 mm long. The dose was approximately 13% of a single dose.

Illumination was with source B, passed through the filters indicated (see fig. 3). Each group consisted of 10 animals.

DISCUSSION

There are described in the literature numerous instances in which radiation from one region of the spectrum ostensibly counteracts or neutralizes the effect of radiation from another region of the spectrum. The tendency to group all such phenomena together under the term "antagonism" seems unfor-

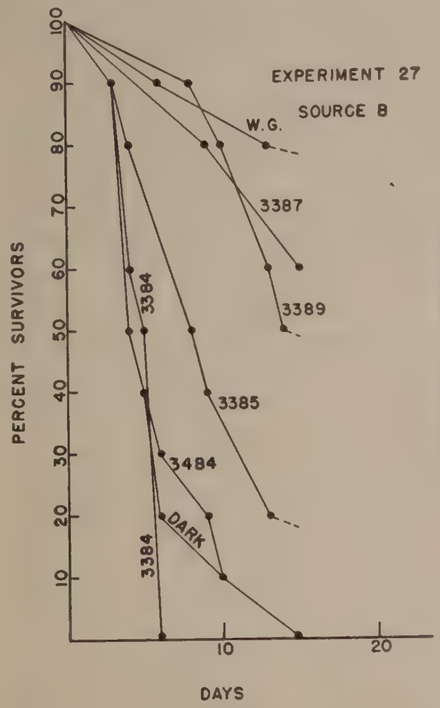

Figure 5

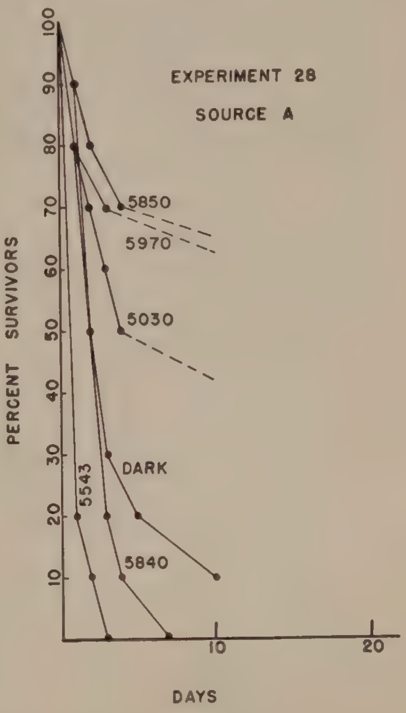

Figure 6

Fig. 5 Survival of larvae of *Amblystoma maculatum* which received repeated doses of ultraviolet radiation. Each dose, which was given 5 days per week, was 6.4×10^5 ergs per cm^2 of mercury arc radiation of wavelengths $0.313\,\mu$ and shorter delivered in about 24 seconds. The larvae were 12 mm long. The dose was approximately 13% of a single lethal dose.

Illumination was with source B, passed through the filters indicated (see fig. 3). Each group consisted of 10 animals.

Fig. 6 Survival of larvae of *Amblystoma maculatum* which received repeated doses of ultraviolet radiation. Each dose, which was given 5 days per week, was 6.4×10^5 ergs per cm^2 of mercury arc radiation of wavelengths $0.313\,\mu$ and shorter, delivered in about 24 seconds. The larvae were in Harrison stage 37 at the beginning of the experiment. The dose was approximately 20% of a single lethal dose.

Illumination was with source A passed through the filters indicated (see fig. 3). Each group consisted of 10 animals.

tunate and may lead to confusion in the interpretation of the underlying mechanisms, which may differ widely. It seems important, therefore, to characterize these phenomena, insofar as possible, so that one may be distinguished clearly from another. Photorecovery or photoreactivation, with which we are concerned here, has a few fundamental characteristics which serve to distinguish it from other instances of ostensible antagonism. The experiments on the widely separated forms, *E. coli, S. griseus, Arbacia* and *Amblystoma,* are sufficiently complete to demonstrate certain common characteristics listed in table 1. In all cases, as is seen in the second column, illumination after ultraviolet radiation results in photoreactivation or photorecovery. This is, of course, the basic observation. From the 4th column we note that illumination has no effect on the normal organism. We note further (column 3) that illumination before the application of ultraviolet radiation has no effect on subsequent recovery. A 4th similarity is that the spectral range for photorecovery is approximately the same in all these cases (column 5). These characteristics alone seem sufficient to characterize photorecovery as a distinct entity. Another point of interest, that the photorecovery process is independent of molecular oxygen (column 6), has only been studied in the case of *E. coli.* In examining the locus of action of ultraviolet radiation and of photorecovery Blum, Robinson, and Loos ('50, '51) have pointed out additional parallelism between the *Arbacia* egg-sperm system and the *E. coli*-bacteriophage system (Dulbecco, '50). This will not be elaborated here, the reader being referred to the original papers.

Such critical experiments as those indicated in table 1 would seem worth extending. There may be difficulty in doing this, to be sure. If one works with a system in which the illuminating light produces some unrelated process the integrated picture may be quite complicated and need further clarifying experiments.[7] Hertel ('04) may have described

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[7] Such difficulty was encountered by Mr. Gordon M. Loos in attempting in this laboratory to demonstrate photorecovery in roots of a higher plant.

TABLE 1

Comparison of certain aspects of photorecovery in four widely separate forms

ORGANISM	ASPECTS STUDIED				
	Illumination *after* ultraviolet	Illumination *before* ultraviolet	Illumination of normal system	Spectral range * of illuminating light	Absence of O_2
Streptomyces griseus	Reactivation [1]	No effect [1]	No effect** [1]	$0.36\,\mu$ to $0.5\,\mu$ [2] (max. $\sim 0.42\,\mu$)	......
Escherichia coli (bacteriophage-host system)	Reactivation [3]	No effect [3]	No effect [3]	$0.3\,\mu$ to $0.5\,\mu$ [2,3] (max. $\sim 0.37\,\mu$)	No effect [3,4]
Arbacia punctulata (fertilized egg system)	Recovery of normal rate of cell division [5]	No effect [5]	No effect [5]	$0.3\,\mu$ to $0.5\,\mu$ [5]	......
Amblystoma	Increased survival and capacity for growth and regeneration [7]	No effect [7]	No effect [7]	$0.3\,\mu$ to $0.5\,\mu$ [7]	......

[1] Kelner ('49); [2] Kelner ('51); [3] Dulbecco ('50); [4] Johnston, Flagler and Blum ('50); [5] Blum, Loos and Robinson ('50; [6] Blum, Robinson and Loos '50, '51); [7] this paper.

* Selection of the spectral range is somewhat arbitrary and a reading of the original papers is recommended.

** Slight injurious effect if any.

photorecovery from the effects of ultraviolet radiation on protoplasmic streaming in *Elodea,* but we cannot be sure that other effects of light were eliminated. The same may be said of Whitaker's ('42) studies on *Fucus* eggs which led him to suggest that the recovery he observed after ultraviolet was due to photosynthesis. A few of the papers discussed by Prát ('36) in his extensive review "Strahlung und antagonistische Wirkung" may have dealt with the type of photorecovery which concerns us here, but in so many of the earlier papers cited by him the experiments are not interpretable because the conditions are poorly defined.

Since Kelner's clear-cut demonstration of the phenomenon, photorecovery after ultraviolet radiation has been described in several forms other than those included in table 1, but has not been so completely differentiated from other effects. We may include the fungi, *Saccharomyces cerevisiae, Penicillin notatum* (Kelner, 49b) and *Neurospora crassa* (Goodgal, '50); the protozoan, *Paramecium aurelia* (Kimball and Gaither, '51);[8] and the sea urchin *Strongylocentrotus purpuratus* (Wells and Giese, '50).

The instances named cover a wide range of living organisms in both the plant and animal kingdoms. We have to conclude that this is a fundamental and widely distributed phenomenon, and this leads us to think of possible evolutionary implications. It seems evident that whatever its nature may be the mechanism for photorecovery has been present in living systems since before the separation of the plant and animal kingdoms, that is for at least a billion years. It is difficult to see how this phenomenon could have adaptive value at the present time in such organisms as the echinoderms or the amphibian larvae, and it seems more likely that it is a vestige of some process that was characteristic of very primitive living systems.

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[8] Studies in this laboratory by T. G. McLellan, Jr., and E. W. Purnell on rotifer eggs, and by E. R. Lindsay and F. C. Wood, Jr., on *Planaria* have indicated photorecovery in these forms although the effects observed were not completely characterized. Studies by H. W. McKay, Jr., and R. L. White suggest very slight photorecovery from sunburn of human skin, but the results are equivocal.

SUMMARY

Illumination with wavelengths between 0.3 μ and 0.5 μ greatly increases the survival time and the recovery of capacity for growth and regeneration of *Amblystoma* larvae that have been subjected to repeated small doses of ultraviolet radiation (λ 0.313 μ and shorter), but only if applied after exposure to the latter. Wavelengths 0.3 μ to 0.5 μ have no observable effect on larvae that have not been exposed to ultraviolet radiation.

Photorecovery or photoreactivation found among bacteria, fungi and echinoderms has these same characteristics, which may serve to differentiate this phenomenon from other examples of ostensible "antagonism."

The mechanism for photorecovery after ultraviolet radiation is widely spread in the plant and animal kingdoms, suggesting that it is a vestige remaining from very early evolutionary times.

ACKNOWLEDGMENT

We are indebted to Prof. Elmer G. Butler for valuable criticism of the manuscript.

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FACTORS INFLUENCING GLYCOGEN SYNTHESIS BY RAT LIVER SLICES IN VITRO[1]

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TEN FIGURES

It is well established that insulin, on the one hand, and certain hormones of the anterior pituitary and the adrenal cortex, on the other, play important roles in the regulation of carbohydrate metabolism. The reports on the action of the pituitary, adrenal, and pancreatic hormones on the hexokinase reaction by Price, Cori, and Colowick ('46) and Colowick, Cori, and Slein ('47) have provided a hypothesis meriting further study. Although the original findings of the Cori group have not been confirmed (Broh-Kahn and Mirsky, '47; Stadie and Haugaard, '49), and the hypothesis that the inhibitory action of the pituitary and adrenal factors are prerequisite for insulin action has been contested (Stadie et al., '51), evidence has accumulated from both in vitro (Krahl and Cori, '47; Villee and Hastings, '49) and in vivo (Stetten and Boxer, '44; Stetten and Klein, '46) experiments which seem to implicate the hexokinase system as one of the sites of hormone action.

We have studied glycogen formation in vitro by liver slices from normal, adrenalectomized, diabetic, and diabetic-adrenalectomized rats. Our results are interpreted as additional evidence in support of the hypothesis that insulin and the adrenal hormones are antagonistic and their regulatory actions are most likely upon the hexokinase system which controls the phosphorylation of glucose. Although our results are accounted for by this interpretation, it is recognized that other sites of action by these hormones are not excluded. In this paper, we shall report our results obtained by chemical and histochemical methods. The histochemical evidence confirms the chemical observations visually.

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[2] This work was done in part during the tenure of a U. S. Public Health Service Postdoctorate Research Fellowship.

EXPERIMENTAL

Animals. Male albino rats of the Wistar strain raised in our own colony, weighing between 200–300 gm, were used. Four groups of rats were studied:

1. Normal rats. These were fasted 18–24 hours before killing.

2. Adrenalectomized rats. The adrenal glands were removed in a one-stage operation under ether anesthesia. The operated animals were fed Purina laboratory chow and given 1% sodium chloride solution as drinking water. They were used 5–7 days after operation. The average weight loss during this period was 10 gm. A thorough post-mortem examination was made in every case, but no gross evidence of adrenal rests was found. From one group of adrenalectomized rats, food was withheld for 16–18 hours before experiments. To another group, a 4–5 gm pellet was left in the cage for the 16–18 hour period prior to sacrifice. This amount of food represents about a third of the animal's average daily requirement.

3. Diabetic rats. Diabetes was induced by intravenous injection of a freshly prepared solution of alloxan monohydrate (5%), at a dosage of 50 mg per kilogram body weight. During the acute stage of diabetes which soon developed, severe glycosuria and hyperglycemia were accompanied by a weight loss up to 30–50 gm in most animals. These rats were used within 7–10 days, and were fasted 24 hours before killing.

4. Diabetic-adrenalectomized rats. Diabetes was induced as above. Within a week after the establishment of diabetes, double adrenalectomy was performed. Thereafter the animals were given 1% sodium chloride as drinking water. The most conspicuous effect of the adrenal ablation upon the diabetic state was an immediate gain of weight. Often there was a further increase in fluid intake and urine output. Though the concentration of sugar in the urine usually showed a decline, the total loss of sugar was not greatly reduced because of the copious polyuria. These animals were used between 5–10 days after adrenalectomy. Blood sugar levels determined before adrenalectomy and before killing the animal were recorded and are reported in table 6.

Medium. The basic incubation medium used had the following ionic composition, K 110, Mg 20, Ca 10, HCO_3 40, Cl 130 mM/L. The substrate used was either glucose, 30 mM/L; pyruvate, 60 mM/L; or glucose 20 mM/L plus pyruvate 20 mM/L. When pyruvate was present, Cl was reduced by an equivalent amount. After equilibration with 95% O_2-5% CO_2, the medium had a pH of 7.5. This medium was chosen because it had been found to be most favorable for glycogen synthesis by rat liver slices.

Procedures. The preparation of liver slices, the experimental procedures and chemical methods for glycogen analysis were those reported by Buchanan, Hastings and Nesbett ('49). The time of incubation was one hour. The reported results are the averages of duplicate flasks. Sample slices were also fixed in a chilled picric acid-alcohol-formalin mixture, embedded in paraffin, sectioned at 5 μ and stained for glycogen by the periodic acid-Schiff technique of McManus. To determine the extent of non-glycogen staining material, control sections were first digested with several changes of saliva for one-half hour (Villee, Deane and Hastings, '49).

RESULTS

Glycogen synthesis by liver slices from normal rats. These results are shown in table 1. It is seen that glycogen was synthesized from both glucose and pyruvate, glucose being slightly more effective as a glycogen-former than pyruvate. The more striking difference, however, was the far greater synthesis of glycogen when both glucose and pyruvate were present than when either alone was present. This increased formation amounted to two to three fold in most experiments. In experiment 89, in which a relatively high level of initial glycogen had resulted a net glycogen breakdown (Hastings and Buchanan, '42) in media containing either glucose alone or pyruvate alone, a considerable net synthesis was obtained in the medium containing both glucose and pyruvate together.

TABLE 1

Glycogen synthesis by liver slices from normal rats

EXP. NO.	INITIAL LIVER GLYCOGEN µG/100 MG WET LIVER	NET CHANGE OF GLYCOGEN µG/100 MG WET LIVER		
		Glucose 20 mM/L	Glucose-pyruvate 20 mM/L of each	Pyruvate 60 mM/L
69	94	82	172	55
71	60	115	205	103
73	33	157	263	131
86	83	63	149	47
88	289	105	295	97
90	112	144	276	139
89 [1]	689	— 100	217	— 144
Average	111.9	117.7	226.7	95.3

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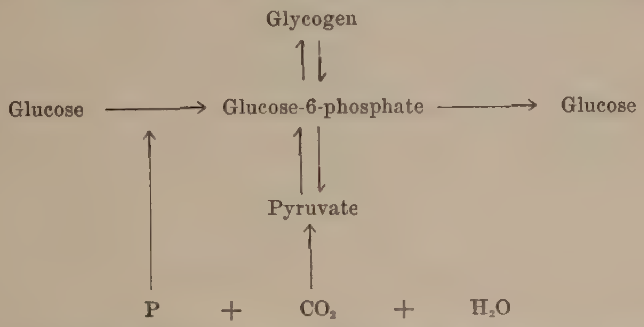
[1] This experiment is not included in the average. Note the relatively high initial liver glycogen level, the net loss of glycogen in media containing only glucose or only pyruvate, and the considerable net increase of glycogen in the medium containing both glucose and pyruvate as substrates.

These results are even more striking considering that the concentrations of substrates represented equivalent concentrations of carbon — whether present as only glucose, only pyruvate, or a mixture of both. The mutually enhancing effect of glucose and pyruvate is probably the result of two primary factors. The first is that in the liver the synthesis or breakdown of glycogen is influenced by the size of the glucose-6-phosphate pool, which in turn is dependent upon the supply and demand of glucose and pyruvate. The dynamic situation is illustrated in the following schematic diagram:



The presence of glucose in the medium not only promotes the formation of glucose-6-phosphate but also reduces its net breakdown, as the phosphatase reaction has been shown to be reversible to some extent by Meyerhof and Green ('49). The presence of pyruvate in the medium provides another source of carbon for glucose-6-phosphate synthesis as well as a substrate for oxidation via the citric acid cycle, and thus would minimize glycolytic and oxidative catabolism of glucose-6-phosphate. The combined presence of glucose and pyruvate in the medium would therefore enhance glycogen formation by the augmented synthesis as well as by the reduced breakdown of glucose-6-phosphate.

The second factor is the generation of high energy phosphate through the oxidation of pyruvate via the citric acid cycle. In the presence of glucose, hexose phosphate is formed from this source of high energy phosphate (Ochoa, '43; Cross et al., '49). The combination of glucose and pyruvate in the medium would thereby lead to greater glycogen synthesis.

Glycogen synthesis by liver slices from adrenalectomized rats. (a) For a period of 16–18 hours before the experiment, one group of adrenalectomized rats was allowed a restricted amount of food, 4–5 gm. This limited ration was not enough to prevent the loss of weight, or to maintain normal levels of liver glycogen at the time of killing. The glycogen formation from glucose, pyruvate, or glucose plus pyruvate by liver slices from these rats is shown in table 2. It is seen that the amounts of glycogen formed from all three substrates were about 50–80% more than those formed by normal livers under similar substrate conditions. (b) In the course of our experiments, we were surprised to find that, if we omitted food completely, liver slices from adrenalectomized rats formed much less glycogen.

TABLE 2

Glycogen synthesis by liver slices from partially fed adrenalectomized rats

EXP. NO.	HOURS OF RESTRICTED FEEDING	WEIGHT LOSS	INITIAL LIVER GLYCOGEN µG/100 MG WET LIVER	NET CHANGE OF GLYCOGEN µG/100 MG WET LIVER		
				Glucose	Glucose-pyruvate	Pyruvate
		gm				
48	17	—8	8	104	323	182
52	15	—6	107	181	273	123
53	18	—4	5	203	425	153
56	16	—24	12	156	351	114
57	16	—7	6	187	252	222
91	18	—12	13	119	316	155
96	17	—3	8	249	399	270
103	16	0	203	242	378	184
Average			45.3	187.6	339.6	175.4

The following experiments were therefore carried out. Paired rats were operated on the same day and kept in the same cage until 16–18 hours before the experiment. Then the animals were put in separate cages, and one rat was given a 4–5 gm pellet while the other was not. They were killed within half an hour of each other. The results on glycogen formation are given in table 3. The effect of adding the pellet of food on the capability of liver slices to make glycogen in vitro is clearly shown.

Histological sections of the incubated slices from two of these experiments (experiments 96 and 97) were prepared and stained for glycogen. The difference in glycogen deposition between slices from the completely fasted (figs. 1, 2, and 3) and the partially fed (figs. 4, 5, and 6) adrenalectomized rats is visually demonstrated.

When restricted feeding versus complete fasting were compared in normal rats, no significant difference in weight loss, initial liver glycogen levels, nor in glycogen formation were observed.

Glycogen synthesis by liver slices in the presence of adrenal cortical extracts. The potency of the adrenal cortical extract (ACE)[3] used is given as 50 dog units or 2.5 rat units per milliliter. The ethanol (10%) contained in the aqueous extract was removed by distillation under reduced pressure, and the extract brought back to its original volume with distilled water. To each experimental vessel containing 2 ml of incubation medium and one liver slice, 0.2 ml of ACE was added. To control vessels 0.2 ml of 0.154 N NaCl solution was added. The results on glycogen formation in the presence or absence of ACE in the medium are summarized in table 4. They show that a sizeable reduction in glycogen formation occurs in the presence of ACE with all three

TABLE 3

Effect of restricted feeding versus complete fasting on glycogen synthesis by liver slices in vitro

EXP. NO.	HOURS BEFORE EXP.	FOOD LEFT IN CAGE	WEIGHT LOSS	INITIAL LIVER GLYCOGEN μG/100 MG WET LIVER	NET CHANGE OF GLYCOGEN μG/100 MG WET LIVER		
					Glucose	Glucose-pyruvate	Pyruvate
		gm	*gm*				
			Adrenalectomized rats				
96–a	17	4	—3	8	249	399	270
96–b	16.5	0	—16	11	54	119	92
97–a	17	4	—4	1410	—60	270	—240
97–b	16.5	0	—10	18	76	122	64
103–a	17	4	0	203	242	378	184
103–b	16.5	0	—12	14	24	78	27

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[3] We express our appreciation to Dr. M. H. Kuizenga of the Upjohn Company, Kalamazoo, Michigan, for the generous supply of the aqueous adrenal extract.

substrates in liver slices from both fasting normal and partially fed adrenalectomized rats. Histochemical preparations confirmed that less glycogen appeared in slices incubated in media containing ACE than in those without added hormone.

TABLE 4

Glycogen synthesis by liver slices in the presence of adrenal cortical extract (ACE)[1] added in vitro

RATS	EXP. NO.	INITIAL LIVER GLYCOGEN µG/100 MG WET LIVER	NET CHANGE OF GLYCOGEN, µG/100 MG WET LIVER					
			Glucose		Glucose-pyruvate		Pyruvate	
			no ACE	with ACE	no ACE	with ACE	no ACE	with ACE
Normal rats complete fasting	51	10	...	...	169	133	...	...
	54	4	...	...	257	153	...	...
	57	49	88	61	...	...	...	...
	60	22	104	60	...	...	...	...
	66	144	122	60	...	...	79	30
	69	94	82	89	172	82	55	34
	71	60	115	115	205	117	103	75
	73	33	157	129	263	208	131	94
	Average	52.0	111.3	85.7	193.2	138.6	92.0	58.2
Adrenalectomized rats restricted feeding	52	107	181	189	273	210	123	82
	53	5	203	124	425	344	153	182
	55	12	156	127	351	318	114	106
	56	6	187	134	252	202	222	196
	68	16	135	119	...	...	101	47
	72	17	155	127	199	178	135	104
	Average	27.2	169.5	136.7	300.0	250.4	141.3	117.8

[1] See text for amount used.

Glycogen synthesis by liver slices in the presence of epinephrine. The adrenal cortical extract used in the above experiments contained not more than 1.2×10^{-4}mg epinephrine per 0.1 ml extract (personal communication from Dr. M. H. Kuizenga). However, since epinephrine has been reported to cause glycogenolysis (Sutherland and Cori, '51), the question arose as to whether the above results could be due to the epinephrine present rather than the cortical hormones of the extract. Experiments were therefore carried out to determine the effect of epinephrine alone at varying concentrations. The epinephrine used was a solution of epinephrine hydrochloride from Parke, Davis and Company. This was diluted to give concentrations of 10^{-2}, 10^{-3}, and 10^{-4}mg per 0.1 ml, and 0.2 ml of the diluted solutions were added to 2 ml of incubation medium. A control vessel with the addition of 0.2 ml saline and another with the addition of 0.2 ml ACE were set up in each experiment. The results show that the inhibitory effect of ACE on glycogen synthesis cannot be accounted for by its epinephrine content. Epinephrine alone in a concentration of 2×10^{-4}mg per vessel (an amount equal to that in the ACE used) did not cause a decrease of net glycogen formation. These results are shown in table 5.

TABLE 5

Glycogen synthesis by liver slices from normal rats in the presence of epinephrine added in vitro

EXP. NO.	INITIAL LIVER GLYCOGEN µG/100 MG WET LIVER	NET CHANGE OF GLYCOGEN, µG/100 MG WET LIVER				
		Control without hormone	Control with ACE [1]	Epinephrine added, mg/vessel		
				2×10^{-4}	2×10^{-3}	2×10^{-2}
74	58	196	149	189	143	82
75	20	179	148	172	179	96
76	60	143	123	138	115	64
Average	46	173	130	166	146	82

[1] The epinephrine content of the amount of ACE used is not more than 2.4×10^{-4} mg per vessel.

Glycogen synthesis by liver slices from alloxan diabetic rats. The liver glycogen of fasting alloxan diabetic rats has been reported to be more than 1000 µg per 100 gm liver (Weber, '46; Morita and Orten, '49). In most of our rats, which were killed within 10 days after the production of diabetes, we have found levels varying between 2600 and

4500 µg % after 24 hours fasting. In our experimental system, a net increase in liver glycogen occurred consistently only in slices with an initial glycogen level lower than 300 µg % and a net decrease of glycogen usually occurred in slices having higher initial levels (Hastings and Buchanan, '42). The liver slices from fasted alloxan-diabetic rats were, therefore, not suitable materials in which to study glycogen formation in vitro. In a few experiments carried out with these animals, a net glycogenolysis was observed in media containing either glucose or pyruvate as substrate. The net loss was usually less in the glucose than in the pyruvate containing medium. In a few instances, a small and probably insignificant net increase occurred in the medium containing both glucose and pyruvate as substrates.

It may be of passing interest to note here that in the acute, severe diabetic state induced by alloxan, the rats lost weight steadily, and on post-mortem examination showed disappearance of depot fat, gross enlargement and marked congestion of the adrenals, and a decrease in size of the spleen. It is known that alloxan diabetes constitutes a severe stress and usually causes adrenal hypertrophy (Applegarth, '49; Eranko, '50). The high glycogen levels found in the livers of our rats may, therefore, have resulted from increased gluconeogenesis through the enhanced activity of the pituitary and adrenal glands.

Glycogen synthesis by liver slices from diabetic-adrenalectomized rats. These animals posed another problem: they could not withstand a fast of longer than 10 hours. This is in agreement with the experience of others (Krahl and Cori, '47; Villee and Hastings, '49). Therefore, a 4–5 gm pellet of food was given during a period of 16 hours before the experiment. On this regime, the initial level of liver glycogen of 12 of 14 rats fell below 0.1 mg %, a level favorable for the demonstration of glycogen formation in vitro. The results of 13 experiments are summarized in table 6.

In graph 1 are plotted the means of net glycogen formation by liver slices of normal, adrenalectomized, and diabetic-adrenalectomized rats from media containing glucose, glucose plus pyruvate, and pyruvate. It is apparent that, while the normal and the adrenalectomized rats formed slightly more glycogen from glucose than from pyruvate, and twice as much from glucose plus pyruvate, the diabetic-adrenalectomized rats formed much less glycogen from glucose than from pyruvate, and just about the same from the mixture as from pyruvate alone. These results are confirmed by histochemical preparations of the liver slices (figs. 7–10).

TABLE 6

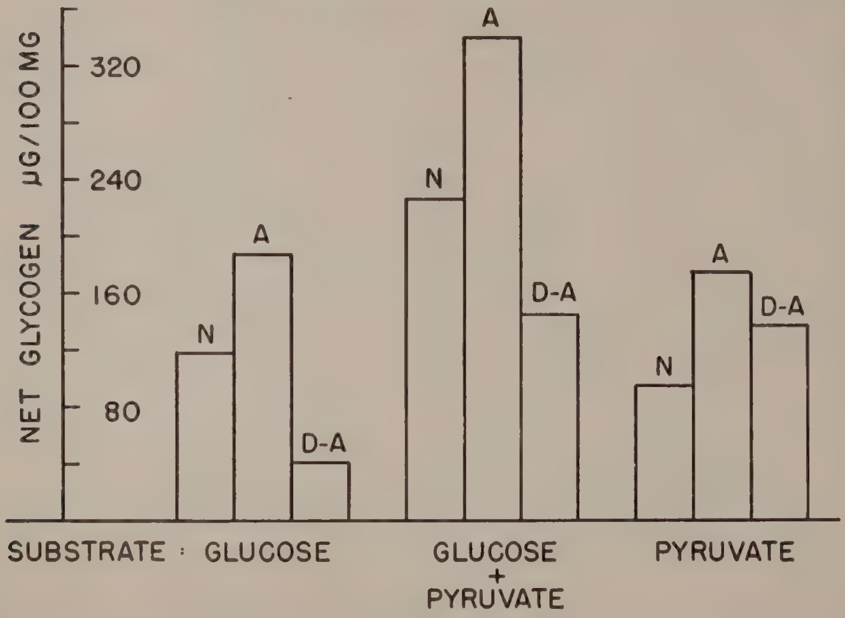
Glycogen synthesis by liver slices from diabetic-adrenalectomized rats

EXP. NO.	BLOOD SUGAR MG %		INITIAL LIVER GLYCOGEN µG/100 MG WET LIVER	NET CHANGE OF GLYCOGEN µG/100 MG WET LIVER		
	Diabetic pre-adx.	Diabetic + adx.		Glucose	Glucose-pyruvate	Pyruvate
127	380	43	71	133	290	260
130	457	124	126	14	169	118
131	594	72	4	27	192	135
132	541	62	0	199	285	226
133	620	215	44	—5	54	91
134	575	...	2	75	168	157
135	557	106	378	62	99	52
136	336	44	10	25	38	57
137	456[1]	50	47	12	128	157
138	380[1]	64	35	—34	124	134
139	298[1]	50	75	15	106	110
140	320[1]	73	45	6	81	137
142	232[1]	73	52	15	148	146
Average			68.4	41.7	144.8	136.8

[1] Fasting blood sugar.

DISCUSSION

The Coris ('27) have observed that "The absence of liver glycogen in fasting adrenalectomized rats is not the result of a disturbance in the synthesis of sugar into glycogen, since liver glycogen is formed when glucose is fed." In our in vitro system, the liver slices are generously supplied with high concentrations of glycogenic substrates. The rate of formation of glycogen is therefore determined by factors other than the supply of gluose or glucose precursors. The formation of more than a normal amount of glycogen by liver slices from adrenalectomized rats suggests that, in the absence of the adrenal hormones, there is an activation or a release of an inhibition of some process concerned with glycogen synthesis in the liver. This is corroborated by the observation that added adrenal cortical extract reduces glycogen formation. The latter finding is in agreement with the results of Verzar and Wenner ('48a, b), Bartlett et al. ('49), and Stadie et al. ('51), but is not consistent with the results of Chiu and Needham ('50). Having a bearing on this point are the observations of Park and Krahl ('49) that injection of ACE further depresses the uptake of glucose by the diaphragm muscle from rats treated with anterior pituitary extract.



Graph 1 The heights of the columns indicate net glycogen (µg/100 mg wet liver) formed by liver slices from normal (N), adrenalectomized (A), and diabetic-adrenalectomized (D-A) rats, from media containing glucose (30 mM/L), glucose plus pyruvate (20 mM/L of each), and pyruvate (60 mM/L).

It is generally accepted that one of the metabolic defects of diabetes is a decreased ability to utilize glucose at a particular blood sugar level. The mechanism of hormonal regulation in bringing about normal glucose utilization is unsettled. The recent observations of Stadie, Haugaard and Marsh ('51) have led these workers to postulate the hypothesis of competitive binding of insulin and pituitary hormone to the tissue as an explanation for the antagonistic effect of these hormones on glucose uptake and glycogen synthesis by rat diaphragm. These and other observations (Perlmutter and Greep, '48) seem to preclude the concept that the presence of inhibitory pituitary factors is a prerequisite for insulin action. Nonetheless, the evidence now at hand seems to confirm the hexokinase system as one of the sites of action of the hormones. Chernick and Chaikoff ('51) have demonstrated two metabolic blocks in the diabetic liver. One of these is concerned with the conversion of glucose to fructose-6-phosphate. This would indicate an insulin effect on the hexokinase and/or isomerase reactions.

In the diabetic-adrenalectomized rats, the insulin deficiency caused by alloxan administration apparently leaves the anterior pituitary hormone(s) unopposed in its inhibitory action on the hexokinase system, even though the adrenal cortex is also removed. Under these circumstances, if the hexokinase hypothesis is correct, one would anticipate a decrease in glycogen synthesis from glucose but normal glycogen synthesis from pyruvate. Such, indeed, are our findings. This is further strengthened by our finding essentially the same glycogen formation from glucose plus pyruvate as occurred from pyruvate alone in livers from diabetic-adrenalectomized animals.

SUMMARY AND CONCLUSIONS

The formation of glycogen by liver slices, in vitro, was studied in a bicarbonate-buffered, potassium rich medium. The substrates used were glucose (30 mM/L), pyruvate (60 mM/L), and glucose plus pyruvate (20 mM/L of each).

Liver slices were obtained from fasting normal, adrenalectomized, alloxan-diabetic, and diabetic-adrenalectomized rats. Chemical analyses for glycogen were made from liver slices before and after incubation.

Livers from adrenalectomized rats formed more glycogen from all three substrates than normal livers. Livers from diabetic animals contained high levels of liver glycogen, despite fasting, and showed glycogen breakdown on incubation. Livers from diabetic-adrenalectomized rats synthesized less glycogen from glucose than normal livers, but slightly more glycogen from pyruvate than normal livers.

Livers from both normal and adrenalectomized rats formed twice as much glycogen from a medium containing both glucose and pyruvate than from media containing only glucose or only pyruvate alone. Livers from diabetic-adrenalectomized animals did not show this increased glycogen formation from the mixture of glucose and pyruvate.

In the presence of adrenal cortical extract added in vitro, livers from both normal and adrenalectomized animals showed decreased glycogen deposition. This effect was shown to be independent of epinephrine at the concentration which was present in the extract.

Histochemical preparations of liver stained for glycogen confirmed the chemical data.

It is suggested that these results are consistent with the hypothesis that insulin and the adrenal cortical hormones exert opposing effects on the hexokinase system.

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PLATES

PLATE 1

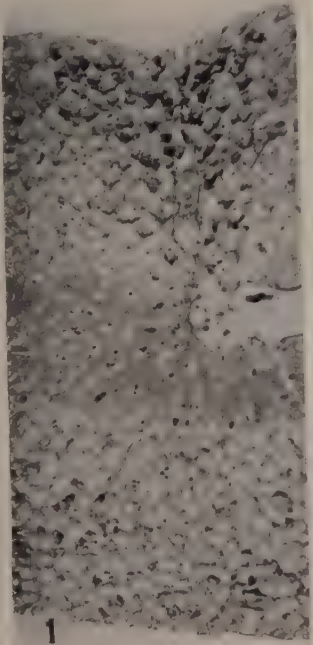
EXPLANATION OF FIGURES

One to three are of liver slices from a rat which was adrenalectomized a week previously and fasted for 18 hours before the experiment began (exp. 96b). Four to six are of liver slices from a rat which was adrenalectomized and fed one 4 gm pellet of chow during the 18 hours before the experiment began (exp. 96a). One and 4, substrate, glucose only; 2 and 5, substrate, glucose plus pyruvate; 3 and 6, substrate, pyruvate only.

The slices were fixed in ice-cold picric acid-alcohol-formalin, sectioned transversely at 5 μ, and stained in the same dish by the periodic acid-Schiff technique. The photomicrographs were taken with a green filter so that the stained material (glycogen and reticulum) appears black. Exposure time, development, and printing were alike for all 6. × 150.

With each substrate, the liver from the partially fed rat has synthesized more glycogen than that from the completely starved rat. Whereas approximately the same amount of glycogen is laid down with glucose or with pyruvate as the substrate, much more is formed when the two are combined. The deposition of glycogen at the periphery of liver slices only was described earlier by Deane, Nesbett, Buchanan and Hastings ('47). In the livers from both rats, initial slices fixed without incubation exhibit no visible glycogen within the liver cells.

CHING-TSENG TENG, F. MAROTT SINEX, HELEN W. DEANE AND A. BAIRD HASTINGS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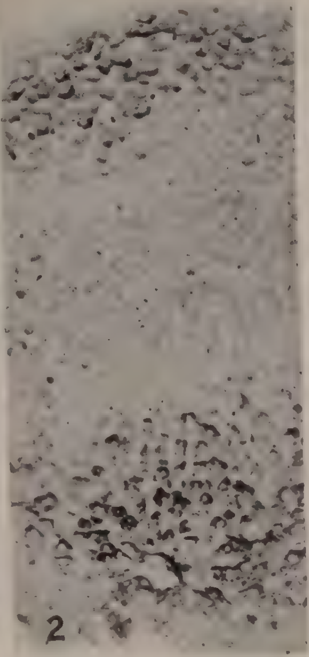
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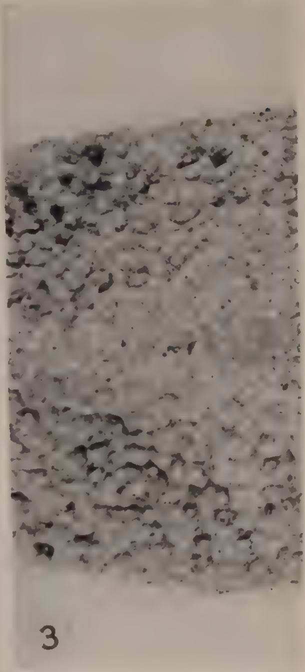
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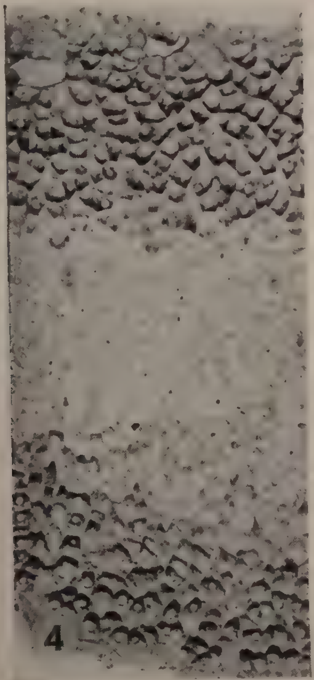
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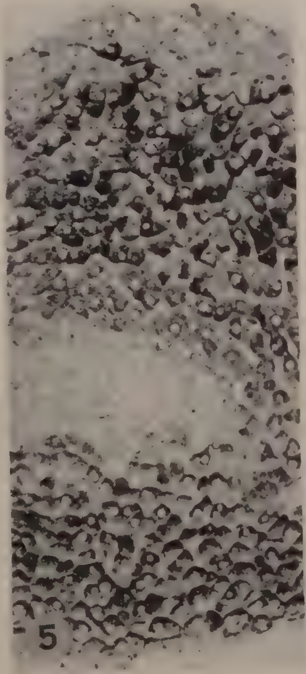
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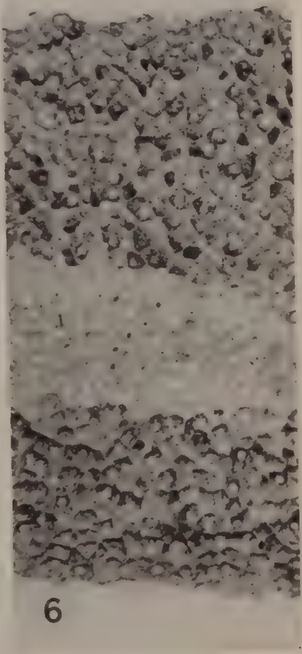
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PLATE 2

EXPLANATION OF FIGURES

Photographs of liver slices from a diabetic-adrenalectomized rat which received one 4 gm pellet of chow during the 18 hours before the experiment began (exp. 139). Seven, control slice without incubation; 8, substrate, glucose only; 9, substrate, glucose plus pyruvate; 10, substrate, pyruvate only. Prepared and photographed as for plate 1.

The control section shows virtually no intracellular material (glycogen) nor does the slice incubated with glucose alone. The slice incubated with glucose plus pyruvate and that with pyruvate alone exhibit similar amounts of deposited glycogen. The non-diabetic rat shows an equal amount of new glycogen with glucose and with pyruvate and increased formation when the two substrates are combined (see figs. 1–6).

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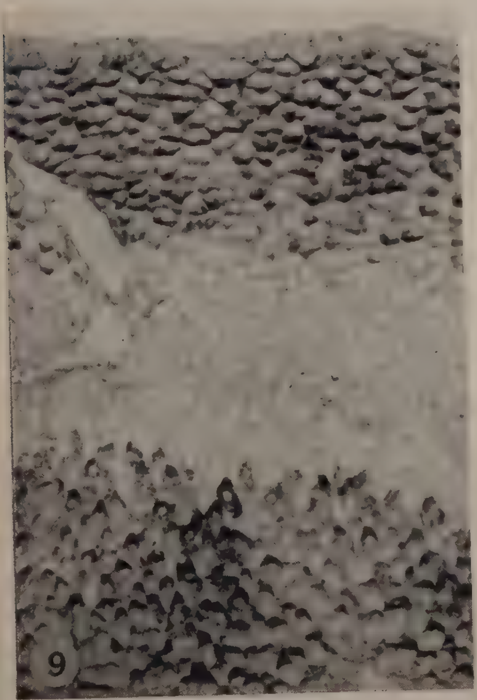
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PROTEIN CHANGES IN TISSUE CULTURE CORRELATED WITH RADIOACTIVE PHOSPHORUS UPTAKE[1]

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THREE FIGURES

The measurement of growth in tissue cultures has been a matter of concern in this laboratory for a number of years (Cunningham and Kirk, '42; Tomkins, Cunningham and Kirk, '47). Hull and Kirk ('50) have studied the metabolism and synthesis of nucleic acids as a means of growth measurement while the uptake of radioactive phosphorus through the culture tube has been used also in this connection (Hull and Kirk, '50). A definition adopted arbitrarily for the meaning of growth in tissue culture involved the increase of mass of the actively metabolizing material (Cunningham and Kirk, '42) and with the additional provision that an absolute increase of any constituent measured over that implanted must occur before it could be stated that growth was positively present.

A constituent which is certainly related to this definition of growth would be the protein of the metabolizing tissue. Whether the content of protein is reasonably constant in the tissue is not known positively though the indications from other studies would indicate a fair constancy of protein content of cells. Davidson and Leslie ('50) have recently measured protein changes with some success. Their technique involved analysis after incubation for a limited period of time in a single medium. The protein content was correlated

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[1] Aided by grants from the American Cancer Society recommended by the Committee on Growth, and the Research Board of the University of California.

with the content of several other chemical constituents. The amount of blood clot used was considerably greater than that reported here. Aside from this investigation, little work has been done in the field. Most culturists utilize large volumes of blood clot which necessarily contain so much protein as to render difficult the evaluation of that contained in the tissue itself. Were it possible to make a clean separation of clot from growing cells the analysis of the remaining cells would be the preferred method for evaluating the protein content. Since this, as yet, is not a practical procedure in spite of some claims made to the contrary it seems desirable to follow the technique used for some time in this laboratory of growing the cultures in as little clotted plasma as possible. The amount of protein contributed by this small amount of clot is a small fraction of the total protein found and, further, it can be kept sufficiently constant that variations in it do not contribute large errors to the over-all analysis. Warner, Hanawalt, and Bischoff ('49) did obtain some protein measurements in the absence of clot by growing their cultures on glass cloth as substrate. They did not obtain any rapid or striking increases in protein content.

The results reported in this present paper represent an extension of work previously done in the evaluation of growth processes in this laboratory. The protein content is followed at variable time periods and correlated with the uptake of radioactive phosphorus as measured through the culture tube (Hull and Kirk, '50).

EXPERIMENTAL

Culture technique

The method of culturing used was essentially that previously described (Signorotti, Hull and Kirk, '50). Chick hearts were taken when 11½ days old and cut in half. Each half-heart was weighed on the quartz helix balance as previously described and cut into approximately 1 mg pieces all of which were transferred into a special roller tube of the type described by Hull and Kirk ('50). This allowed estimation of the P^{32} uptake and was adaptable to the analysis for protein. The tissue fragments were held in place by the coagulum from 0.1 ml of plasma which had been diluted with 9 parts of Tyrode's solution corresponding to 0.01 ml of original plasma. The plasma used was a dried preparation obtained from the Difco Laboratories as specified by the Tissue Culture Association. It was reconstituted with the correct amount of sterile water before dilution with Tyrode's solution.

The *media* were as follows: (1) Control Tyrode's solution prepared with a four-fold increase of glucose and reduction of sodium chloride to 7.7 gm per liter; (2) Forty per cent horse serum diluted with 60% Tyrode's solution; (3) Twenty per cent embryo extract prepared as previously described diluted with Tyrode's solution; (4) "Standard medium" consisting of 40% horse serum, 20% embryo extract, and 40% Tyrode's solution. Tracer phosphorus diluted to contain 20 μc per milliliter was added to give a concentration of 0.2 μc per milliliter. This concentration has proven not to be harmful to the tissues and to allow a sufficiently rapid emission for successful counting. All tubes were kept at controlled pH by treating them with a mixture of 5% carbon dioxide and 95% air and maintaining them under this gas mixture.

Analyses were performed for P^{32} as described by Hull and Kirk ('50). The tubes were counted every 24 hours and the medium was changed every 48 hours along with pH adjustment. Analyses for protein were carried out by a modification of the biuret method similar to that of Weichselbaum ('46). The reagent used was that of Weichselbaum but some modification of his technique was found necessary with this material, namely, an extraction of the fat prior to analysis. Fat obtained in tissue cultures invariably produced cloudiness in the colored solutions. Before analysis, the medium was poured from the tubes which were then rinsed with normal saline and allowed to drain vertically for 5 minutes. To each tube was added 3 ml of saline and the tube was stoppered and rotated for an hour in the incubator to remove water soluble substances, notably protein carried in the clot. The washing, rinsing, and draining was repeated as before. Finally, to each tube was added 15 ml of 3 to 1 alcohol-chloroform mixture which was allowed to stand in the tubes for 24 hours. That solvent was then removed and the tubes drained for half an hour. The preparation was then ready to dissolve in 3% NaOH. The cultures were dissolved by standing at room temperature for 24 hours in 1.5 ml of 3% NaOH. The solutions and washings of the culture tubes were transferred to graduated centrifuge tubes using a transfer pipette equipped with rubber bulb. Pouring was impractical because of the acquisition of charred organic material from the flamed mouths of the tubes. Solutions were made to 4 ml volume in the centrifuge tubes and centrifuged to remove bubbles which were quite persistent unless this treatment was adopted. One milliliter of Weichselbaum's reagent was then added and the tubes stoppered, shaken, and centrifuged for 10 minutes. After centrifuging, the solutions were filtered through Buchner 0000 microfunnels into small test tubes. Full color development was obtained in one to two hours at room temperature or one-half hour at 37°C., room temperature normally being used.

Numerous auxilliary checks of the biuret procedure were made by means of the Kjeldahl analysis and agreement was found between the two procedures. This is in accordance with numerous other investigations (Kingsley, '42; Hiller, Greif, Beckman and Plazin, '48).

RESULTS

Two major sources of difficulty were encountered early in this investigation. One of these resulted from the fact that at times blood clots took up exceptionally large amounts of tracer phosphorus in the absence of tissue. This could also occur in some cultures in a similar manner. The general use of clot blanks is ordinarily avoided because of the feeling that the tissue culture is in some way distinctly different from that of a clot system alone. This investigation demonstrated early that it was essential to determine the uptake of tracer phosphorus by the clot alone in order to ascertain whether some unusual condition such as contamination or an abnormal batch of serum or other effect was present.

The other difficulty encountered was due to the occasional great increase of protein in the clot blank. At times the blanks determined on clots increased greatly over 24-hour periods to amounts considerably greater than that occurring in the culture itself. Both of these sources of difficulty had to be placed under control. It was shown that the presence of growing tissue in the clot prevented such deposition of protein. The amount of protein obtained with variable amounts of clot was essentially constant as long as tissue was present.

The study of protein increase in growing cultures expressed in terms of the amount of implanted tissue produced the data shown in figure 1. It will be noted that embryo extract and standard medium are both satisfactory media for tissue culture growth. This is in agreement with the findings of Hull and Kirk ('50). It is interesting that embryonic extract showed actually a higher amount of protein formed than did standard medium. Tyrode's solution alone and the 40% horse serum medium were about equally unsatisfactory. Both curves are characterized by a considerable though slow diminution of the amount of protein.

Similar comparisons of the same cultures in terms of uptake of tracer phosphorus are shown in figure 2. The results here were in essential agreement with the values for protein except that throughout most of the culture period embryo extract was not as good as standard medium. The difference between the protein value and the uptake of tracer phosphorus was notable. Furthermore, with horse serum medium there was a distinct though not large rise in the uptake of phosphorus. The contrast is quite noticeable between this value and that found for protein change with time. The percentage values of figure 2 are those of cultures terminated at the times shown rather than being carried for the full period as is customary with tracer uptake measurements. This was necessitated by the protein analysis which caused termination of the culture.

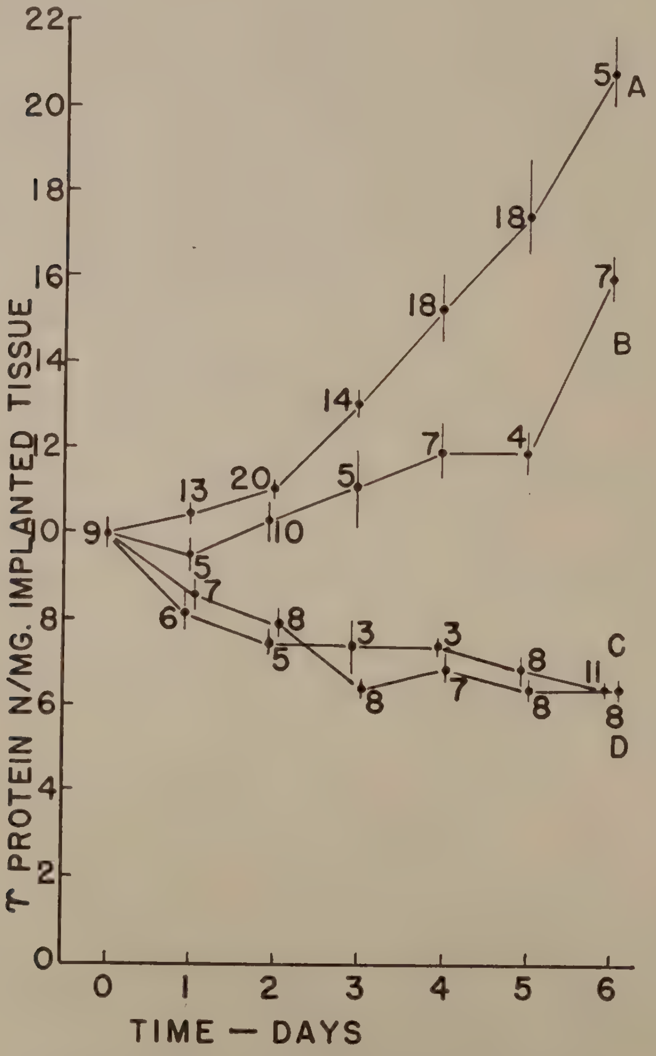

Fig. 1 Protein nitrogen changes with time.
A, embryonic extract. B, standard medium.
C, horse serum. D, 40% horse serum.

This procedure led to a certain amount of irregularity in the curves which is obviated by the use only of 6-day cultures in which P^{32} uptake was measured regularly but all cultures were terminated for protein analysis at the end of 6 days.

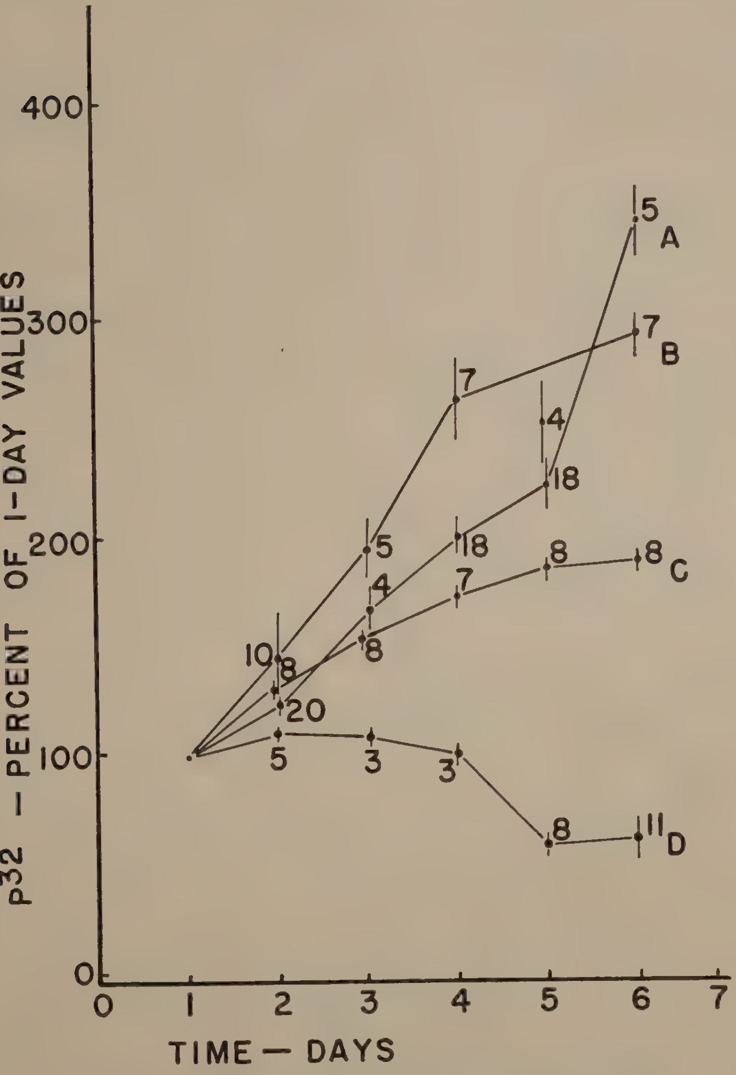

Fig. 2 P^{32} uptake in percentage of one day values.
A, embryonic extract. B, standard medium.
C, Tyrode's solution. D, Tyrode's solution.

These particular data are shown in figure 3 in which the curves are not only smoother but are in somewhat better agreement with the protein increase values. They still show the same fundamental characteristics as the curves of figure 2.

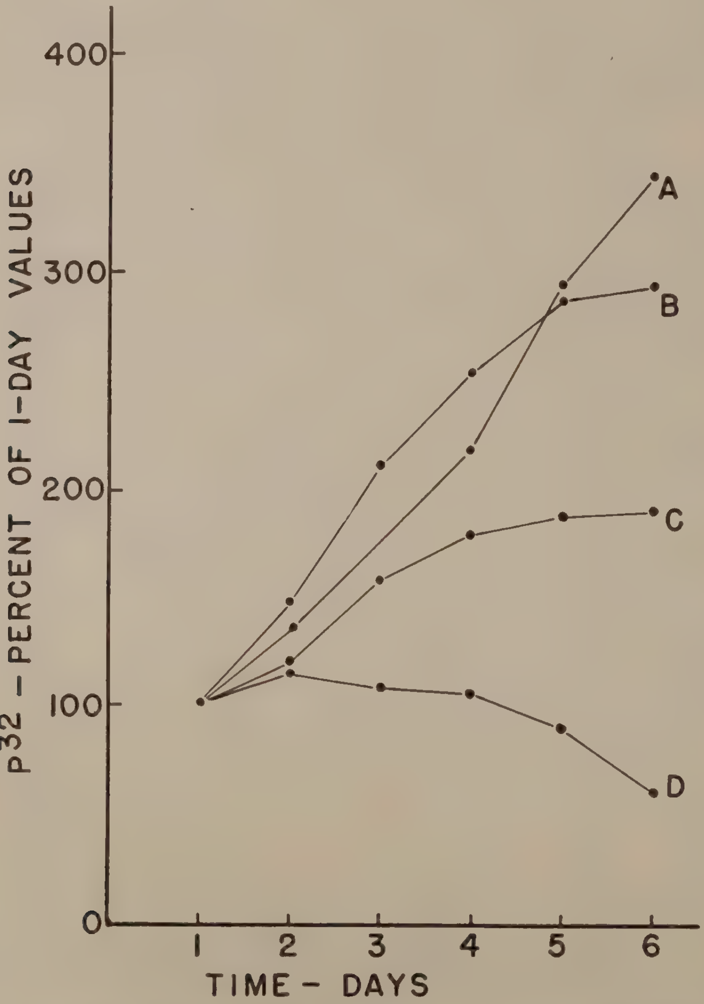

Fig. 3 P^{32} uptake in percentage of one day values. Serial measurements on those cultures terminated at 6 days.

A, embryonic extract.	B, standard medium.
C, horse serum.	D, Tyrode's solution.

DISCUSSION

Examination of the data which are shown graphically in the accompanying figures reveals a number of interesting and important points which are related to the interpretation of the nature of tissue growth and development. If the arbitrary definition of growth adopted in this work be accepted, it is clear that the protein content does not indicate true growth in the absence of embryonic extract in any instance, nor does the presence of serum have any beneficial effect. This is no longer indicated when the data on uptake of tracer phosphorus is examined. Here it appears that some growth may have occurred in the presence of serum but none in Tyrode's solution alone. That we are dealing here with different aspects of the tissue behavior can scarcely be doubted. Microscopic observation of the cultures in Tyrode's solution alone and in the serum medium offers the clue to interpretation. In neither type of culture was there any cell migration after three days, with a scant migration (area increase) in both during the first two to three days. After the migration ceased, the cells in Tyrode's solution alone rounded up and gave the appearance of dead cells, while those in serum medium retained the appearance of living but inactive cells.

It is common to observe some migration in Tyrode's solution cultures, and with this must go some metabolic turnover. The slight initial uptake of P^{32} may well be due largely to this restricted chemical activity. The gross picture is necessarily one of loss as indicated by the rapid diminution of protein. Serum addition evidently has the effect of extending the minimal metabolic activities of the cells for a longer time without allowing new synthesis of proteinaceous constituents, but maintaining longer and at a higher level the chemical activities which result in uptake of phosphorus. The role of the implant in supporting whatever activity occurred appears to be significant, particularly when Tyrode's solution alone is present. Fibrin clot cannot be the source since there is sufficient of this material to support much more extended activity than is ever observed. The serum carried in the plasma producing the clot could have only the slight effect of that small amount of serum, which must be negligible with respect to protein synthesis but might be responsible for the small uptake of P^{32} in Tyrode's solution.

The marked effect of embryonic extract in producing protein synthesis, even as compared with complete medium, may be interpreted as indicating that the processes which produce protein synthesis are supported by the embryo extract alone in contrast to those which result in uptake of P^{32} which are obviously supported to a limited but definite extent by serum. It cannot be stated that this is a sufficient explanation for the reverse order of the two growth evaluating methods, in one of which complete medium appears to be superior and in the other of which embryo extract gives the better results throughout most of the growth period. This does not seem to explain the consistent reversal of the curves in both figure 2 and figure 3 in the time interval between 5 and 6 days. At this growth interval, the clots showed a tendency to undergo lysis. This was retarded by the presence of serum which also appeared to inhibit the amount of migration, and probably of cell proliferation. This difference in behavior of serum-containing and serum-free media appears to offer the best explanation of the effect.

It will be noted that the curves for P^{32} uptake, particularly that with Tyrode's solution medium, are not in good agreement with earlier data of Hull and Kirk ('50). The cause of the discrepancy is clear. In the earlier work, control of pH was exclusively by the buffer initially added. In Tyrode's solution, alkalinization of the medium resulted from loss of carbon dioxide, and it was shown in this investigation that alkalinization always leads to binding of phosphate by constituents of the culture. The use of a controlled gas mixture containing 5% carbon dioxide throughout the entire growth period eliminated this effect with blanks, and led to a lowering of the measured P^{32} uptake values in this medium. The presence of sufficient protein and additional buffer in the more complete media of the early experiments did not allow any large deviation of pH. The effect may be that reported by Hanks ('48) who showed precipitation on the solid culture components of calcium phosphate when alkalinization occurred.

Davidson, Leslie and Waymouth ('49) rotated their cultures in Tyrode's solution for 20 hours, considering the tissue to be in a "resting state" after this time. In terms of protein content, it appears from the data of this investigation that no such resting state is to be expected since the protein falls off rapidly and immediately except when growth-promoting medium is present. The constancy they observed with fractions of nucleic acid phosphorus is not in agreement with the loss of protein. It appears that they ordinarily used only two cultures per point which may cast some doubt on the validity of their statistics with as variable a system as the tissue culture. It is also not at all impossible that some constituents in the culture may remain essentially constant even though others are diminishing rapidly. Restoration of the latter constituents to the deficient culture system might be interpreted as growth even though it failed to return to the original amount, and this effect would not have been noted in the experiments quoted because of the method of study.

Parker ('36) apparently first claimed that a serum-Tyrode's solution medium was a true maintenance medium in that cells remained viable in it for long periods of time. White ('49) also produced a protein-free medium that had a similar effect though the period studied was much shorter. While no long-term studies are reported in this paper, it may be noted that there are indications in support and explanation of the maintenance effect of serum-Tyrode's solution. It is shown that the protein content of cultures in this medium diminishes rapidly for the first three days but then reaches a rather constant plateau. Microscopic observation shows the cells to have an entirely healthy appearance, and the P^{32} uptake shows them to be metabolically active. Neither of these two findings is present when Tyrode's solution alone composes the culture medium. The cells appear to be dead or dying at the end of 6 days and the P^{32} uptake is markedly below the initial values.

It is not possible to compare directly our results with embryonic extract and complete medium with those of Davidson and Leslie ('50) because of the difference in experimental procedure. These authors showed a falling ratio of protein content to desoxyribonucleic acid phosphorus content (DNAP) during a 6-day period. Comparing the results for protein content found by us with the DNAP found by Hull and Kirk ('50) the ratio of protein content to DNAP actually rose in embryo extract cultures and was diminished only slightly with complete medium. It might be concluded that the ratio tended to remain constant in the 5- to 6-day period. Including also the results with horse serum and with Tyrode's solution cultures, there is still essential parallelism between the protein content and the DNAP. This relationship may be taken to indicate that the protein content is essentially constant in the cell, a point of great interest if protein content is to be employed in evaluation of tissue growth. It appeared possible that incubation of cultures for 24 hours in serum-Tyrode's solution medium after various periods in growth promoting medium as practiced by Davidson and Leslie ('50) was responsible for loss of protein. To test this possibility, protein was analyzed before and after washing for 24 hours with serum-Tyrode's solution. An average of 10.1% protein nitrogen was lost. This loss falls far short of accounting for the nearly 50% reduction in ratio of protein content to DNAP observed by the above authors. It is unlikely that the difference arises from unusually low protective action of the serum-Tyrode's solution medium, though the composition of the latter was not indicated.

SUMMARY

The protein content of cultures of embryonic chick heart fragments was studied in relation to the constituents of the

culture medium, and in comparison with the uptake of tracer P^{32} at various time intervals.

Embryo extract medium was found to give rise to the greatest amount of protein synthesis, not equalled by a complete medium containing also horse serum. Both Tyrode's solution and a mixture of this with horse serum led to a marked diminution of protein over that implanted.

Protein synthesis and uptake of P^{32} tended to parallel each other but not completely so. The relation of complete medium and embryo extract medium was reversed in their effects. Horse serum medium led to consistent but small uptake of tracer phosphorus as contrasted with its effect on protein synthesis.

The interpretation of the observed facts in terms of the role of implant and the protective action of serum on the tissue is discussed.

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A FURTHER STUDY OF THE LOCAL RESPONSES OF AXONS

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EIGHTEEN FIGURES

In an earlier paper from this Department, Rosenblueth and Luco ('50) studied some of the properties of the local responses (see Hodgkin, '38) that develop at the cathode when rectangular pulses are applied to mammalian myelinated axons. That study was mainly concerned with the responses to pulses of brief duration (less than 1 msec.). The records were taken directly from the cathode, without balancing the passive effects of the applied currents. These effects thus obscured some of the features of the responses.

In the present study a method was devised that largely or entirely eliminates in the records the component due to the electrotonic distribution of the impressed e.m.f., and thus allows an accurate estimate of the changes of the membrane potential that occur in response to the stimuli. The method was used to study the effects of rectangular pulses, cathodal and anodal, and those of alternating currents, rectangular or sinusoidal. This paper deals with the responses to relatively long rectangular pulses. The other studies will be reported in subsequent communications.

METHOD

The nerves used were spinal roots, removed from cats anesthetized with dial (0.65 cm^3 per kilogram, intraperitoneally). The spinal canal was opened from L_2 to L_6. The dura was cut at the midline. The roots employed were dorsal or ventral, from L_5 to S_2. They were cut beyond their emergence from the dura and at their spinal insertions. They were placed in Ringer's solution in a Petri dish, and strands of different diameters were isolated under a dissecting microscope. The strands were usually relatively large; often a complete ventral or dorsal root was employed. The two ends of the strands were crushed and ligated with threads that allowed the attachment of the nerve to two of the electrodes.

These electrodes were relatively long chlorided silver wires, immersed in Ringer in glass tubes. The lower end of the tubes was filled with agar-Ringer. The agar continued beyond the tubes, covering a narrow, flat cotton wick. The nerves lay on top of the edges of some of these wicks. Other electrodes could be placed from above, utilizing for contact the lower edge of the agar-covered wicks. Electrodes of this type are quite impolarizable, because of the large silver-silver chloride interface; they have a low resistance (about 1,000 ohms) because the agar bridge is short and has a relatively large cross-section area; and they make a relatively localized contact with the nerves, about 0.5 mm, since the thin edge of the flat wicks is used for this contact.

The nerve and electrodes were immersed in mineral oil. The experiments were carried out at room temperature, from 20 to 25°C.

Two or three model III-C Grass stimulators, coupled appropriately, provided all the rectangular currents employed. The output of each stimulator was converted into radio-frequency a.c. and fed to the primary of a transformer (Grass ''stimulus isolation unit''). The envelope of the high-frequency train from the secondary, after rectification, yielded the original wave form, no longer connected to ground. The polarity of the pulses or trains could thus be reversed without changing the ground connection of the nerve. The output of the stimulators was delivered to the nerves through series resistances of 10,000 ohms. This ensured the independence of the different stimuli when several stimulators were used.

In all the experiments one of the stimulating electrodes made contact with one of the crushed ends of the nerves. We shall refer to the d.c. pulses as cathodal or anodal, depending on whether the other stimulating electrode, on live tissue, was the cathode or the anode. Repeated tests showed that when spike discharge occurred in response to the anodal shocks the impulses were always initiated at the anode, and not at some spurious cathode near the crushed end. The records were always of the monophasic type, i.e., with one of the leads on the other crushed end.

In the majority of the experiments the amplifiers were resistance-capacity coupled, with an over-all time constant of 0.5 sec. Observations made with a direct-coupled amplifier yielded results similar to those obtained with the capacity-coupled units. The input was always made through a push-pull cathode-follower stage, coupled directly to the following stage. This type of input has a very high impedance, and thus provides quite independent records when several are taken simultaneously; in addition, it minimizes the introduction of spurious ground connections into the circuit. All the stages in the amplifiers were of the push-pull type.

The usual recording set-up consisted of three single-beam cathode-ray oscillographs, with their corresponding amplifiers. The local effects were photographed from one of them. In the others the presence or absence of propagated responses could be observed, or else the amplitude of the stimuli could be measured. In some observations a two-beam Electronic Tube Corporation oscillograph was used. One of the beams recorded the local changes while the other showed the currents delivered to the nerves.

RESULTS

A. The balancing of the applied currents. The local potential changes are of small amplitude in the records, usually less than 0.2 mv., much smaller than the differences of potential set up by the currents applied. Although it is possible to record the active local changes directly at the cathode or anode of the stimulating electrodes (see Hodgkin, '38; Rosenblueth and Luco, '50), it is desirable and convenient to minimize in the records the changes directly attributable to the stimuli.

In the present experiments the differences of potential between the recording electrodes due to the e.m.f. impressed were considerably reduced by the method diagrammatized in figure 1. A pulse applied through electrodes *a* and *b* estab-

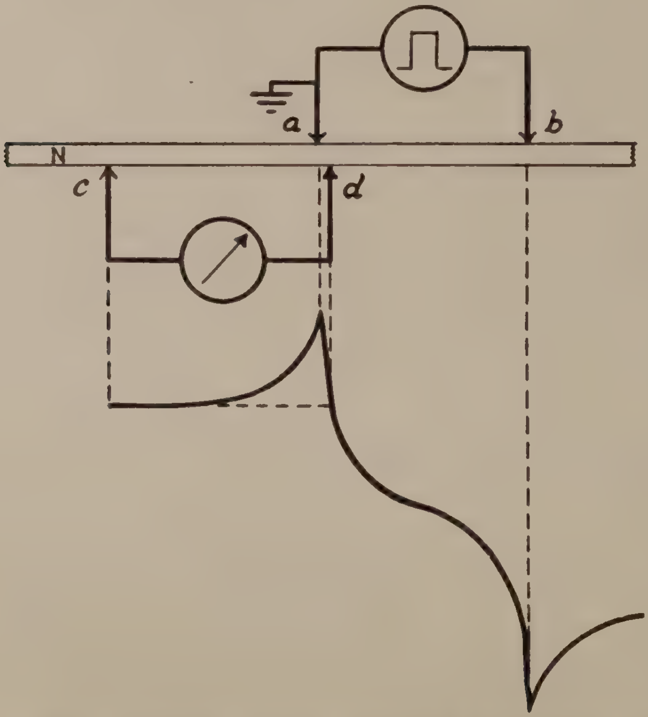

Fig. 1 Diagram illustrating the method employed to minimize the passive electrotonic potentials set up by currents applied to nerves. At the steady state of the passage of a rectangular pulse applied to the nerve N through the electrodes *a* and *b* the approximate distribution of potential will be as shown by the curve at the bottom of the diagram. By moving the recording electrode *d* along the nerve it is possible to find a position that will make it isopotential with electrode *c*. In practice both electrodes *b* and *c* were at the crushed ends of the excised spinal roots.

When describing the position of the electrodes in the different experiments, reference will be made to the letters in this diagram.

lishes the potential gradients shown at the bottom of the diagram. It is clear, therefore, that by moving the recording electrode *d* within the interpolar stretch it is possible to find a point of contact which is isopotential with the point *c*. It is also clear that as electrode *d* crosses the critical point its difference of potential with regard to *c* will reverse in sign.

The diagram in figure 1 represents the steady state of the passage of d.c. and indicates that a perfect balance should be obtained if the nerve behaved like a passive conductor. A practically perfect balance was in fact usually found with weak d.c. after equilibrium was reached. At the make and the break of d.c., however, the balance was never total. In addition, the balance position shifted when the intensity of the pulses was changed.

In practice, it was always easy to locate the critical position for electrode *d* by moving it along the nerve with a micrometer. It was usually about 0.3 mm from the pole, in the interpolar region, and shifts of less than 0.2 mm away from that position in either direction resulted in marked unbalance.

Figure 2 shows the electrotonic distribution of a weak pulse along the lower edge of a nerve, in the vicinity of the overlying cathode. The curve in this figure differs significantly from the usual representation of the electrotonic distribution in the extrapolar region. This difference is due to the fact that in the classical observations the amplitude at the pole is measured actually from that pole, whereas in the present records it was measured at the point of the nerve opposite the pole. In this region the nerve cannot be regarded as a unidimensional conductor, but is in fact a volume conductor. It was invariably found that the potential difference measured at the pole was much greater (about 10 times) than that measured at the underlying electrode. This discrepancy appeared even when relatively fine strands were dissected from the roots.

In the records from which figure 2 was constructed there were only minimal local responses, since the pulses were about one-fifth the intensity of the cathodal threshold pulses. In figure 3 another series of observations is illustrated, but with pulses of just threshold intensity when cathodal. The records are complex, they exhibit different waves both during the passage of the currents and afterwards. An important feature of these records is that whereas their main, quasi-

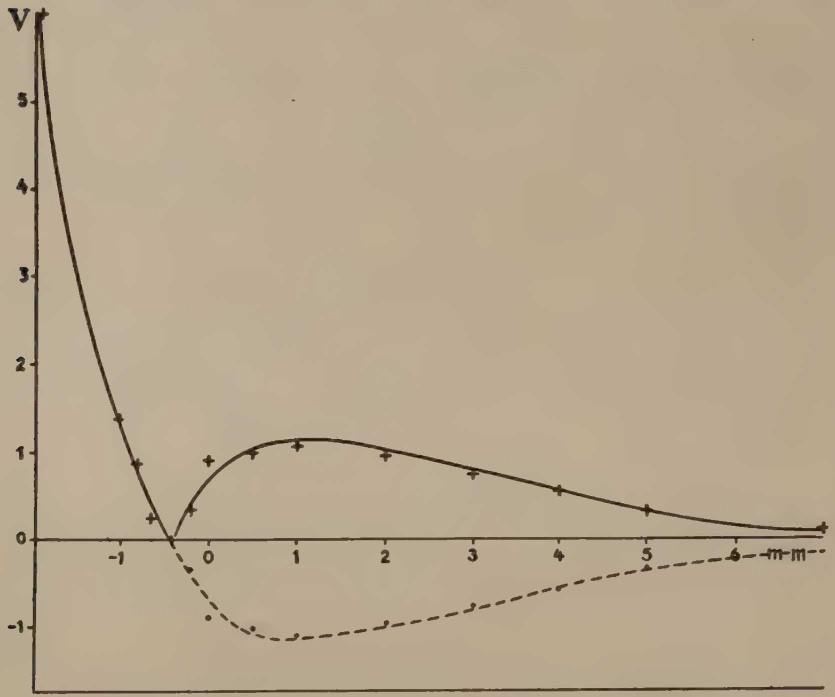

Fig. 2 Typical electrotonic distribution of a weak pulse along the lower edge of a nerve in the vicinity of the overlying cathode. A 5-msec. pulse with strength 0.20 threshold was applied at a rate of 10 per sec., first with cathode at electrode *a*, and then with the reversed polarity. The recording electrode *d* was moved to the positions indicated by the abscissae; the point opposite electrode *a* is taken as O, and positive distances correspond to the extrapolar stretch. The ordinates show the maximum amplitude (in conventional units) of the potential changes in the records of the cathodal pulses. The solid line shows the absolute value of this amplitude, with no consideration of the polarity. The dotted line shows the reversal of sign which actually took place. The curve corresponding to the anodal pulses was quite similar to that illustrated here.

rectangular component reversed in sign as the exploring electrode crossed the region of balance, the other waves did not reverse. Thus, in the cathodal records there is always an initial negative wave (R) and at the end of the pulses there is a positive swing (S). Conversely, in the anodal records an initial positive swing (S) develops and subsides, and at the end of the pulses a prominent negative wave (R) is obvious. For reasons that will be detailed later we attribute these oscillations to changes of the membrane potential. We shall designate the excursions labeled R as cathodal or anodal local responses, and those labeled S as postcathodal or anodal positive swings, respectively.

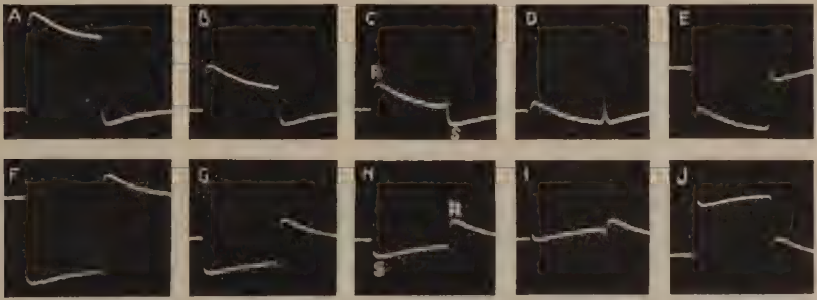

Fig. 3 Changes in the records of the effects of cathodal and anodal pulses due to shifts of electrode *d*. The observations were carried out as in figure 2. The pulses had a duration of 10 msec. and their intensity was just below the threshold of the most excitable fibers when the cathode was at *a*. Upper records: cathode at *a*; lower records: anode at *a*. The successive pairs of records correspond to equidistant shifts of electrode *d* beginning just below the pole (A and F) and ending at a point about 0.5 mm away, in the interpolar region (E and J).

Note that while the quasirectangular main component in records A and F gradually decreases and then reverses its sign, the waves labeled R and S in records C and H do not reverse.

In these and the following cathode-ray oscillograph records upward excursions denote negativity of electrode *d* with respect to electrode *c*. The time scale is calibrated by the duration of the pulses; the on and off of these pulses appear as sharp artifacts.

B. The measurement of the local responses and positive swings. Since the method of recording employed balances out some of the components of the potential changes elicited by the pulses, it is necessary to adopt a conventional base line for the measurement of the potential fluctuations that appear in the tracings. The base line we adopted was the nearly flat region that follows the initial positive swing when long anodal pulses are applied. In other words, the balance was adjusted so that this flat region coincided with the resting potential level. The amplitude of the several waves mentioned was then measured from this base line.

C. The influence of the intensity of the pulses. The fluctuations of the membrane potential vary strikingly and in a nonlinear fashion when the stimuli are intensified progressively. As shown in figure 4, with weak pulses the fluctuations are minimal or absent; as the pulses become stronger the waves become increasingly prominent. In general the anodal and cathodal records are not mirror images; the local responses are more ample than the positive swings.

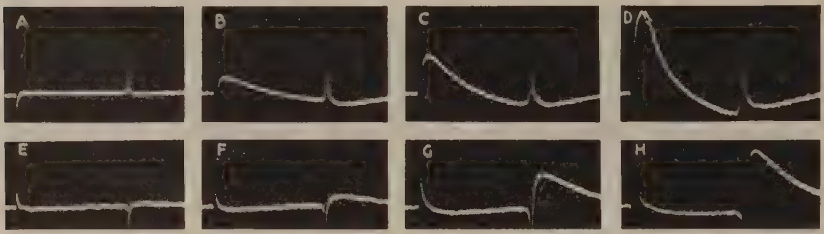

Fig. 4 Effects of varying the intensity of the pulses on the local responses and swings. Records with electrode *d* in the position of optimum balance. A to D: cathode at *a;* E to H: anode at *a*. Intensity of the pulses (with the threshold for the cathodal pulses as unit): A and E, 0.1; B and F, 0.45; C and G, 0.75; D and H. 1.

Figure 5 illustrates graphically the changes of amplitude of the anodal local responses to pulses of three durations, as a function of the intensity of these pulses. A similar curve, convex toward the abscissae, was found by Arvanitaki ('39), by Rosenblueth and Luco ('50) for the corresponding observations on the responses to brief subthreshold cathodal pulses, and in the present study for those to long cathodal pulses.

The positive swings exhibit a different behavior when the stimuli are gradually intensified. They increase more rapidly at first, and later more slowly, so that the curves correlating the amplitude of the swing with the intensity of the stimuli are concave toward the abscissae.

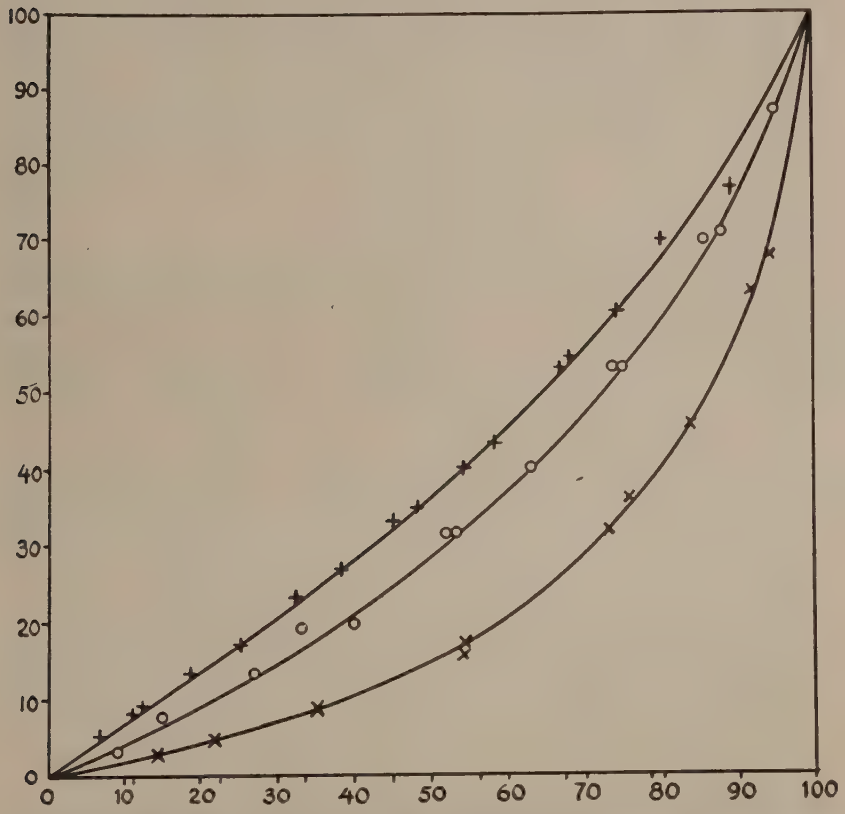

Fig. 5 Amplitude of the anodal local responses as a function of the intensity of the pulses. Abscissae: intensity, with threshold taken as 100 units. Ordinates: amplitude, with that corresponding to a just threshold shock taken as 100 units. Upper curve: 20-msec. pulses; middle curve: 2-msec. pulses; and lower curve 0.5-msec. pulses.

D. The influence of the duration of the pulses. It is desirable to describe this influence separately for the different waves under consideration.

With brief shocks (0.2 msec. or less) the cathodal local response outlasts the stimulus; indeed, the peak of the response may occur some time after the end of the shocks (see Hodgkin, '38; Pumphrey, Schmitt and Young, '40; Katz, '47; Rosenblueth and Luco, '50). In these cases the responses are diphasic; a positive wave of small amplitude follows the negative excursion. With shocks of intermediate duration (0.5 to 2 msec.) the response ceases at the end of the pulse and is promptly followed by a positive swing, usually of small amplitude. With longer shocks, as illustrated in figure 4, the response declines while the pulse is still flowing through the nerve.

The previous description applies to shocks of various durations and with intensities such that they are slightly below the threshold of the most excitable fibers. If the intensity is fixed at threshold for long shocks and the duration is then gradually decreased, the cathodal local response decreases, to become almost negligible for the brief pulses.

Figure 6 illustrates the influence of the duration of anodal pulses of fixed subthreshold voltage on the corresponding local responses. The responses increase with the duration and seem to tend to an asymptotic maximal amplitude.

The amplitude of the anodal positive swing is not affected by the duration of pulses of fixed voltage that are longer than its own time course. If the shocks end before it has developed it is curtailed; and if the end occurs during its rising phase its amplitude will decrease accordingly.

The postcathodal positive swing behaves much like the anodal response when pulses of different durations are applied. Its amplitude increases and tends to reach an asymptotic level.

E. The distribution of the local responses along the nerve. For these measurements relatively long pulses were applied; the electrode *d* was moved to different positions, as in the observations illustrated in figures 2 and 3. The anodal local response and the postcathodal swing were measured in the records from the resting base line. The anodal swing and the cathodal responses were in turn measured from the potential level that obtained at the end of the anodal pulses, taken with a positive and a negative sign, respectively.

Figure 7 illustrates the amplitude of the responses recorded at different distances from the pole. It is clear that although the local responses are smaller at the position of balance than at that opposite the pole, the difference is not large.

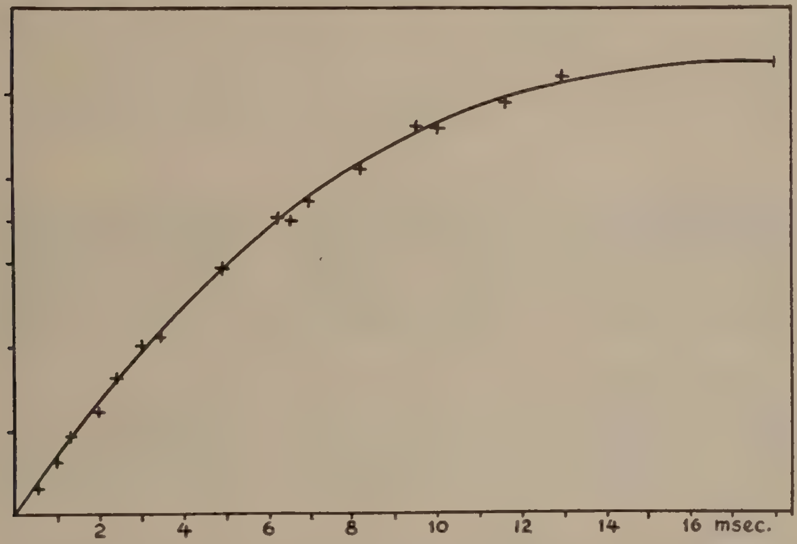

Fig. 6 Amplitude of the anodal local responses as a function of the duration of pulses of fixed intensity. The intensity was adjusted so that it was just threshold with a 20-msec. pulse. Abscissae: durations in milliseconds. Ordinates: amplitudes in conventional units.

F. The changes of the local responses during the refractory period. For these observations, in addition to the relatively long subthreshold cathodal and anodal pulses, brief maximal stimuli were delivered to the nerve through electrode *b* and an additional one (cathode) intermediate between *b* and *d*. These stimuli were delivered at different times with regard to the long pulses.

In general it may be stated that the local responses and positive swings decrease during the refractory period, and that the decrease is the more marked, the earlier the time of development of the corresponding wave with regard to the preceding conditioning spike. Figures 8 (C and F) and 9 A illustrate typical results. It is interesting to note in figure 9 A that the cathodal responses decrease far more than the anodal during the refractory period.

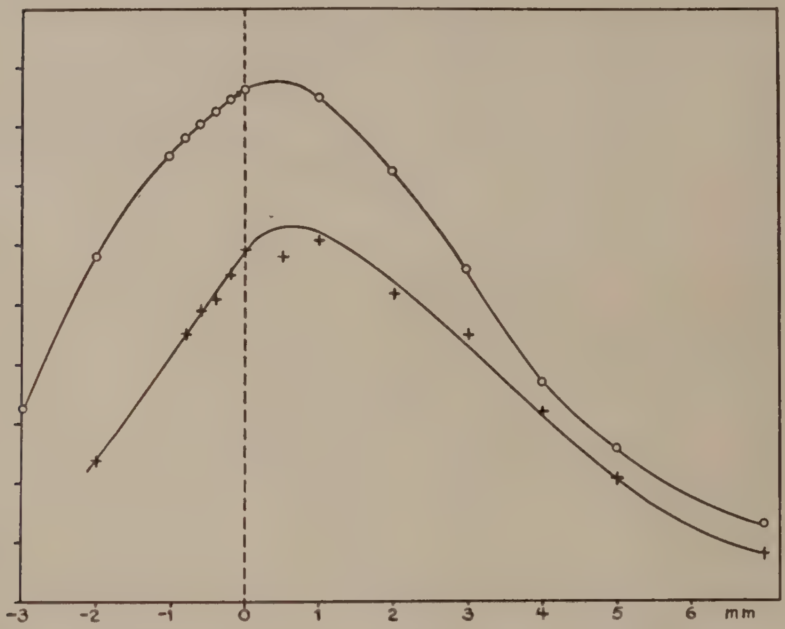

Fig. 7 Distribution of the local responses along the nerve. Abscissae: positions of electrode *d* measured as in figure 2. Ordinates: amplitude of the local responses, in conventional units, with the same scale for both curves; for details on the measurement of these amplitudes see text. Lower curve: cathodal local responses. Upper curve: anodal local responses. Both pulses were just threshold for the most excitable fibers; the durations were 5 msec. for the anodal and 20 msec. for the cathodal.

G. Local effects of threshold stimuli that outlast the development of the spike potential. The monophasic records of the potential changes that follow a maximal stimulus may differ when the recording electrode on intact nerve is at some distance from the site of initiation of the impulse and when it is near this site. In the records taken after propagation over some millimeters, and in those taken near the cathode of brief cathodal shocks, if the amplification is not sufficient to exhibit the afterpotentials, the spike potential is merely followed by a positive deviation which is not due to the arrival of the impulse to the injured end (see Lorente de Nó, '47)

If the stimuli are anodal, and the recording electrode is near the site of initiation of the impulses, the positive deviation mentioned above decreases and is followed by a later negative wave (see lower tracing in fig. 9 B), which increases with the intensity or duration of the anodal pulses, even after they are maximal. This wave is a residual local effect of a strong anodal pulse that outlasts the development of the spike potential.

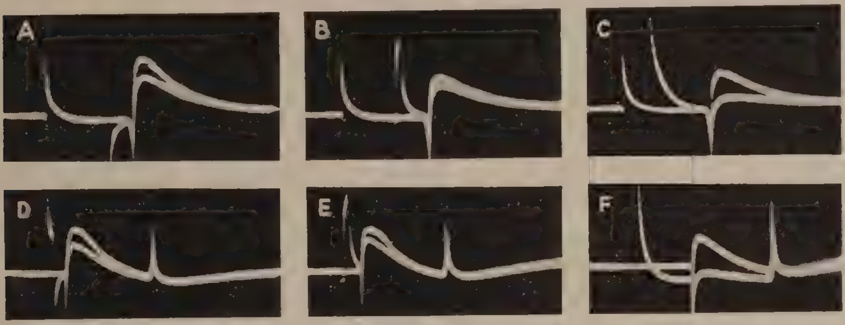

Fig. 8 Similarity of the changes in the anodal and cathodal local responses when conditioned by subthreshold anodal or cathodal pulses or by spike discharge. Test 10-msec. pulses were delivered through electrodes *a* and *b*, with the anode (A, B and C) or the cathode (D, E and F) at *a*. The effects of an additional 1-msec. conditioning pulse delivered through the same electrodes on the local responses to the test shocks were recorded by superimposing the pictures of the unconditioned and conditioned responses.

A and D A subthreshold anodal pulse applied shortly before the development of the local responses results in their increase.

B and E A subthreshold cathodal pulse results in a decrease of the responses.

C and F During the refractory period consequent to the application of a conditioning shock that elicited spike discharge of about 50% of the fibers the local responses are practically abolished.

That the wave in question is probably the remainder of the anodal local response is suggested also by the following observations. If brief subthreshold test shocks are applied during its development, through the same electrodes used for the supramaximal anodal stimuli, the changes of the corresponding local responses are quite different from those reported in the previous section. Instead of the decrease that occurs during the refractory state, an increase of the test responses is observed (fig. 9 B), so that quite ample local effects can be recorded well within the refractory period in these conditions.

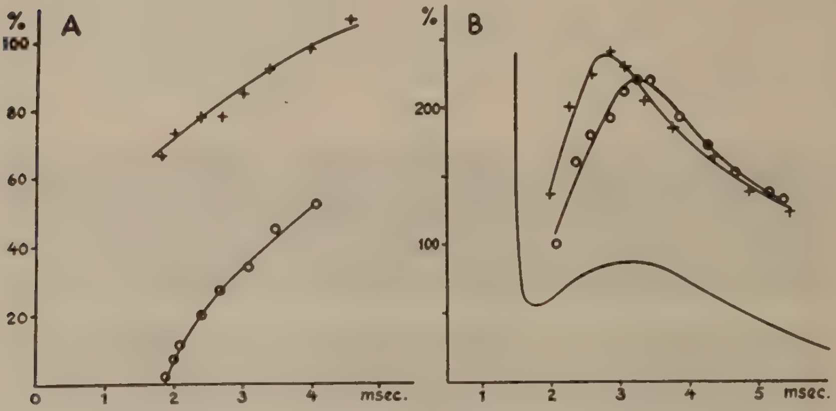

Fig. 9 A Changes in the amplitude of the local responses during the refractory period. Ordinates: amplitudes measured as per cent of the control. Abscissae: interval between the time of application of the brief maximal conditioning stimuli and that at which the peak of the local responses to 1-msec. test pulses took place. Upper curve: (crosses) anodal, and lower curve (circles) cathodal local responses.

B Increase of test local responses during the development of an anodal local response that outlasts the spike potential. Conditioning and test shocks applied through electrodes *a* and *b*. The conditioning stimuli were anodal and supramaximal for the A fibers of the nerve. The lower curve shows the descending branch of the maximal spike and the subsequent potential changes elicited by the conditioning stimuli; the resting potential level is the axis of the abscissae. Ordinates, abscissae, crosses and circles as in figure 9 A.

II. The local responses to threshold cathodal or anodal stimuli. The initiation of spike potentials is always preceded by the development of a local response. It is thus important to study the amplitude and duration of the responses that reach the threshold of spike discharge.

This study was carried out for cathodal shocks of different durations by Rosenblueth and Luco ('50). It was shown that brief shocks require the development of more ample local responses for spike discharge than do long shocks, but that the area of the threshold responses to long shocks is greater than that of the responses to brief stimuli.

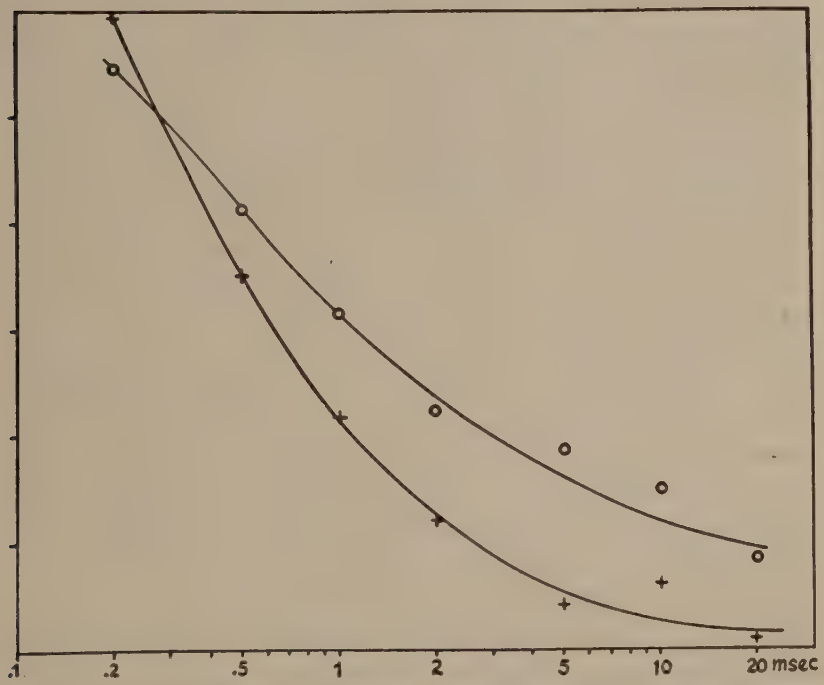

Fig. 10 Local responses to threshold anodal stimuli of various durations. The stimuli were adjusted so that about 1% of the A fibers discharged. Abscissae: durations of the pulses in milliseconds (logarithmic scale). Ordinates: amplitude (crosses) and area (circles) of the responses.

In the present study the threshold responses to anodal pulses of durations ranging from 0.1 to 50 msec. were recorded. As shown in figure 10, both the amplitude and the area of the responses decrease as the pulses are lengthened.

I. The responses to brief test shocks applied during the development of local responses or positive swings. For these observations a long (10 to 50 msec.) cathodal or anodal pulse was given 5 to 20 times per sec. Through the same pair of electrodes, and with the same rate, a brief (0.5 to 2 msec.) shock was delivered at different times before, during or after the passage of the long pulse. Both shocks were subthreshold. The records were taken with electrode *d* at the position of optimum balance. In some of the experiments both shocks had a fixed intensity. Since they summed algebraically, the total degree of positivity or negativity developed by the brief shocks was different when they were applied during the time of flow of the long pulses than when they preceded or followed these pulses. In other experiments, therefore, the intensity of the brief shocks was adjusted so as to add or subtract that of the long pulses when the two coincided.

The results of the two types of experiments are illustrated in figure 11. It is clear that the local responses to the brief pulses increase when they take place during the development of the similar responses to the long pulses, and that they decrease when the positive swings of the long pulses develop. It is also clear, in addition, that during the phase that follows the cathodal local response and the anodal positive swing, the responses to the test shocks decrease or increase, respectively, even though the changes of the membrane potential are slight or minimal at those times.

In these experiments one explores not only the influence of the long pulse on the responses to the brief shocks, but also the converse influence of the brief shocks on the responses to the long pulses. From this standpoint the results were as follows. In confirmation of the reports of Pumphrey, Schmitt and Young ('40) and of Rosenblueth and Luco ('50) it was found that a brief subthreshold cathodal shock depresses the local response to a long cathodal pulse applied a few milliseconds later (see fig. 8 E). On the contrary, if the brief shock is delivered shortly (e.g., 0.2 msec.) before the long one, the two local responses sum and may lead to spike discharge.

The influence of brief anodal shocks on the local responses to subsequent cathodal pulses will be described in section J.

Brief cathodal shocks always depress the local responses to long anodal pulses (see fig. 8 B), even when they precede the end of these long pulses by a fraction of a millisecond. Increases are only seen if the end of the anodal pulse takes place at the time of application of the brief shocks, or coincides with its beginning. Brief anodal shocks have the opposite effect; they cause an increase of the local response

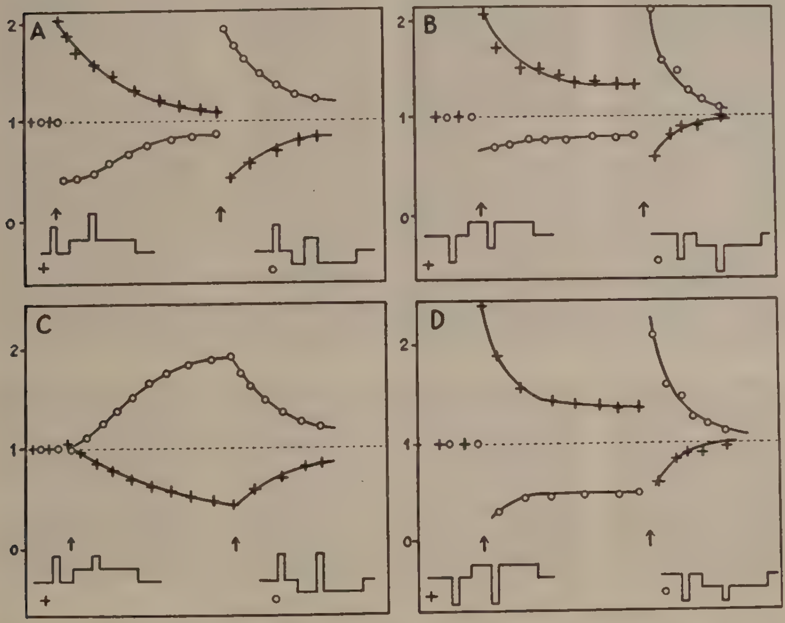

Fig. 11 Changes of the local responses to brief test shocks when they develop in the course of or after the application of long pulses (10 msec.). All the pulses were delivered through the same pair of electrodes. Abscissae: time; the arrows indicate the on and the off of the long pulses. Ordinates: amplitude of the responses measured from the base line imposed by the passage of the long pulses; the amplitude of the control responses is taken as unit. Crosses: long cathodal pulses; circles: long anodal pulses.

A and B The intensity of the brief pulses was not changed when they were superimposed on the long ones, so that the two summed algebraically (see lower diagrams). In A the test pulses were cathodal and in B they were anodal.

C and D The intensity of the brief pulses was adjusted so that their absolute intensity remained constant (see diagrams). C, cathodal, and D, anodal test pulses.

to the long anodal pulse (fig. 8 A), the more striking the shorter the interval between the shocks and the end of the pulses.

J. Interaction of the effects of two brief pulses. An anodal pulse that immediately precedes a brief cathodal test stimulus can either depress or enhance the local response to the latter. If we begin with a brief weak anodal pulse it will depress the test local response (cf. fig. 12 A and H). If we then gradually intensify it, the depression will at first increase, but later it will be less striking and will finally be replaced by an enhancement, the more striking the stronger the anodal conditioning pulse.

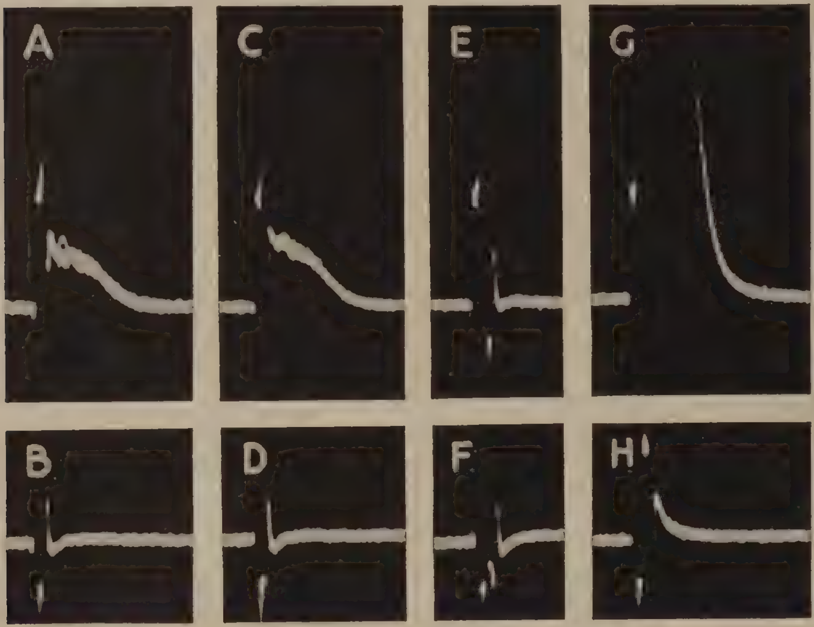

Fig. 12 Interaction of the effects of two brief pulses. Pairs of successive shocks, with duration of 0.2 msec., delivered through the same electrodes.

A and B First pulse delivered alone, cathodal and anodal respectively. C. and D: as in A and B, but second pulse. E: first cathodal and second anodal. F: both anodal. G: both cathodal. H: first anodal and second cathodal.

As the anodal pulses are made longer, the depressing effects become less prominent, while the enhancing influence is more striking. Thus, in figure 13, an anodal pulse of fixed intensity decreased the local response to a cathodal pulse when the former was brief (B), but increased the test response when it was lengthened (D to F).

If a brief cathodal pulse is immediately followed by an anodal one the local responses to the two are diminished. It is thus clear that the cathodal pulse depresses the response to the anodal shock, and conversely, that the anodal pulse decreases or cancels the local response to the cathodal stimulus (fig. 12 E). If this stimulus is liminal, the anodal pulse usually prevents the initiation of impulses. This anodal depression is also seen if the first shock is liminal and anodal; the second anodal pulse will then delay the initiation of the impulses.

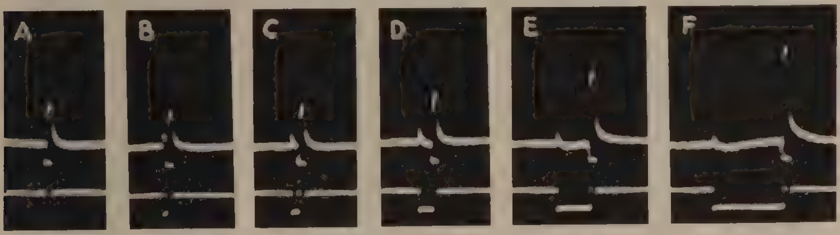

Fig. 13 Influence of the duration of an anodal pulse on the effects of an immediately following cathodal pulse. Upper tracings, electrograms, and lower tracings records of the pulses. The test cathodal pulse had a constant duration of 1 msec.; A shows its effects when delivered singly, and B to F the changes produced by the previous delivery of an anodal pulse with the following durations: 0.5, 1, 2, 5 and 10 msec.

K. The responses to trains of pulses. Since, as shown above (see fig. 8), the development of a local response may modify that of a subsequent one, it was expected that the successive responses to a train of cathodal or anodal pulses would not be constant, and that the differences between them would depend on the intensity and duration of the pulses and on their frequency, that is, on the duration of the intervals between them. These expectations were confirmed. A detailed analysis of the influence of each of the factors mentioned was not undertaken. The following general observations were made, however, and they are of interest for the explanation of some of the topics considered in the Discussion.

As a rule the successive local responses to a train of cathodal pulses decrease until they reach an equilibrium (see fig. 14 G and H). This decrease is in accord with the fact, mentioned before, that the response to the second of a pair of cathodal pulses is smaller than the control response, unless the interval between the two pulses is very brief.

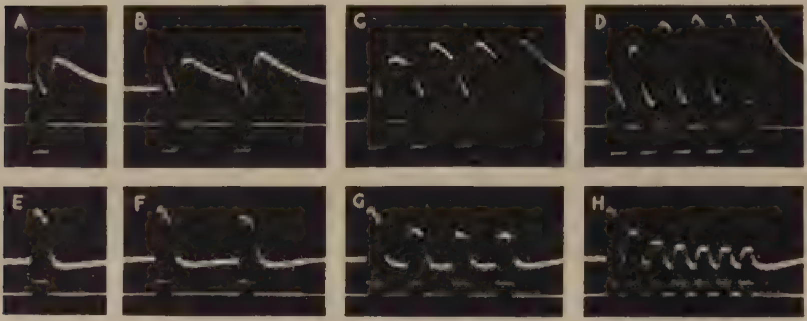

Fig. 14 Responses to trains of pulses. Records as in figure 13. A to D, trains of anodal pulses (2 msec.) with increasing frequency. E to H, cathodal pulses of the same duration.

With very brief pulses, e.g., 0.2 msec., separated by brief intervals, e.g., 0.1–0.2 msec., the responses to 4 successive cathodal shocks are seen to increase and sum, and if the intensity is appropriate, spike discharge may only occur if the 4 shocks are delivered, but not if only three are applied.

As shown in figure 14 C and D, the successive local responses usually increase if a train of anodal pulses is given. Here again, spike discharge may only occur after several stimuli have been applied.

L. The responses to rectangular alternating currents. The behavior of the responses to alternating anodal and cathodal pulses depends on the relative amplitude and duration of the two pulses in each cycle, and on the phase of the cycle, at which the alternating current begins.

Figure 15 illustrates the importance of the phase at the start of a train of alternating rectangular pulses. The greater the fraction of the anodal pulse with which the train begins, the larger the first response. Thus, in A, where the train began with the cathodal half cycle, spike discharge occurred only at the third cathodal pulse; in C, where the train began with the anodal half cycle, the first cathodal pulse elicited spike discharge of some fibers.

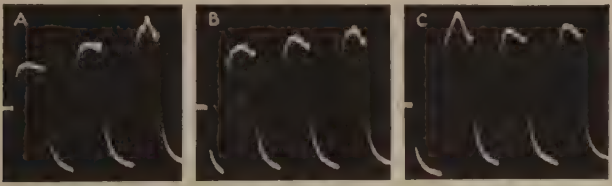

Fig. 15 Influence of the local responses of the moment in the cycle at which a train of alternating rectangular current is initiated. Trains of alternating cathodal and anodal pulses of equal intensity and duration (5 msec.). A begins with a cathodal half-cycle; B with one-half of an anodal half-cycle; and C with the beginning of an anodal half-cycle.

Figure 16 shows the changes due to increasing the amplitude of the anodal pulses interposed between constant cathodal pulses. The local responses grow increasingly, and in D they reach sufficient magnitude to cause spike discharge upon the application of the 4th cathodal pulse. In both figures 15 and 16 it is impossible to separate the anodal from the cathodal local responses; they sum and merge to constitute a single negative deflection.

DISCUSSION

I. The recording method. For the interpretation of the records it is important to consider the source of the potential changes that are observed after balancing out some components by the method illustrated in figure 1.

When a d.c. pulse is applied to a nerve, several factors may contribute to the potential differences that develop between the leads. The on and the off of the pulse appear as sharp large excursions, which are in part genuine constituents of the distribution of the field along the nerve, but which are also partly artifacts due to a capacitative coupling between the stimulating and the recording systems. These rapid excursions need not concern us here.

The impressed e.m.f is distributed in the nerve according to the geometry and the conducting properties of the tissue. This distribution includes transverse and longitudinal components. Insofar as the nerve behaves like a passive linear conducting element this distribution will attain an equilibrium a short time after the current begins to flow and will remain constant until it ceases.

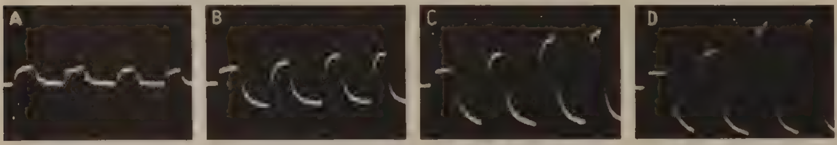

Fig. 16 Influence of the intensity of the anodal half-cycle on the responses to alternating rectangular currents. A: train of cathodal pulses. B to D increasing positivity applied between the same cathodal pulses.

But nerves are not passive linear elements. The externally applied e.m.f. gives rise to local changes of the potential difference which normally exists across the axon membranes; and these changes are not linear: they vary with the duration and intensity of the pulses and they also vary during the time of application of a constant external e.m.f.

The method employed here balances out the steady components, both longitudinal and transverse, of the electrical field that attends the steady flow of current. It thus cancels all the components that correspond to the behavior of the nerve as a passive linear conductor, and may also cancel some active changes. Since the polarity of the local responses and swings does not change when the exploring electrode *d* (fig. 1) crosses the point of optimum balance (see fig. 3), it is clear that these fluctuations are due to local changes of the axons at the poles and in the neighborhood of these poles. It is reasonable to attribute them to changes in the potential drop across the membrane in those regions.

Cole and Curtis ('41) measured the potential differences between two microelectrodes placed across the membrane of the squid giant axon when d.c. pulses were applied from the region surrounding the external microelectrode to a distant region. These differences were greater when the explored region was polarized anodally than when it was polarized cathodally, for any given intensity of the pulses. They inferred that the impedance of the membrane is greater for the inward than for the outward flow of current. This conclusion was supported by the direct measurements of Cole and Baker ('41) of the impedance of the membrane during the flow of currents. In the observations of Hodgkin ('38), Pumphrey, Schmitt and Young ('40), Hodgkin and Rushton ('46), Katz ('47), and Rosenblueth and Luco ('50), as well as in the present study, the reverse relations were found, i.e., the potential drops were greater during the flow of a given cathodal pulse than they were if the pulse was anodal (see fig. 4). In the present study results similar to those of Cole and Curtis were only obtained in nerves to which cocaine was applied (see fig. 17 E and F).

If we consider only the main steady component, excluding the local responses and swings, long pulses (10 msec. or longer) that are below threshold when cathodal have approximately the same point of balance, whether they be cathodal or anodal. This fact indicates that the rectifying properties of the membrane do not play a significant role in the distribution of the field, in the conditions in which the present experiments were done. It does not appear reasonable, therefore, to attribute the responses and swings to rectification.

Although it is possible to conceive passive models for the axon membrane that would lead to potential fluctuations during and after the passage of d.c. pulses similar to those seen in nerves, the view that the local responses and swings denote active changes of the membrane potential is supported by the following data.

(a). The fluctuations decrease or disappear during the refractory period (section F). *(b)* The amplitude of the responses to brief test shocks is importantly influenced by the concurrent or previous application of conditioning pulses. Thus, the responses to the test shocks vary markedly when these shocks are delivered in the course of the passage of long pulses (fig. 11). Again, the response to a given cathodal shock is decreased or increased when the test stimulus follows a previous anodal pulse (figs. 12 and 13). Finally, the successive responses to trains of cathodal or anodal pulses can vary significantly in amplitude (fig. 14). *(c)* All the fluctuations disappear temporarily if the nerve is reversibly damaged by procedures that do not modify importantly its impedance, as shown by the electrotonic distribution of the test pulses applied. As examples of these procedures may be cited the application of strong d.c. or a.c., or of cocaine (fig. 17). *(d)* The local responses to brief cathodal shocks may long outlast the end of the stimuli (fig. 12). *(e)* Two successive local responses may sum if the stimuli are applied in rapid succession, and this summation is greater than linear.

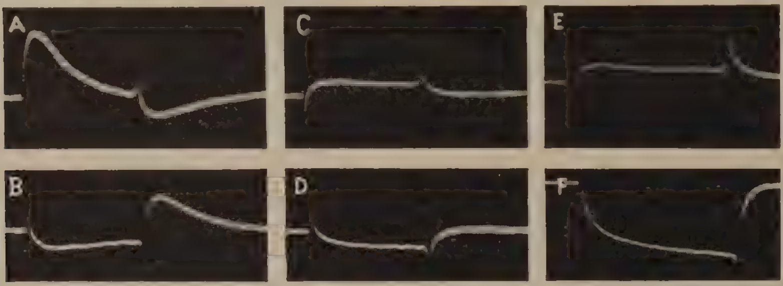

Fig. 17 Depression of the local responses by cocaine. Upper records, cathodal, and lower records, anodal pulses. A and B, controls (10-msec. pulses). C and D, after the local application of cocaine to a segment of the nerve including the electrodes *a* and *d*. E and F, as in C and D, but with stronger and longer (20 msec.) pulses.

This argument is particularly convincing when an initial weak pulse, that leads to a minimal response, greatly enhances that to a test stimulus applied shortly after the first (see Rosenblueth and Luco, '50).

We are thus led to conclude that the local responses and swings are due to local active changes of the membrane potential that occur at and near the poles of d.c. pulses. Following Hodgkin's ('38) suggestion, we call the negative changes local responses. It is only for convenience that we have designated the positive changes as swings, although according to this conclusion they could also be referred to as positive responses, anodal and postcathodal, respectively.

II. A comparison of the cathodal and anodal local responses. The two responses have many features in common. They both occur when current flows outwards through the axon membrane. The two may sum with each other, e.g., when a brief shock is applied in the course of the development of the local response to a pulse of the opposite polarity (see fig. 11). When long cathodal and anodal pulses are employed the time course of the two responses is approximately the same (figs. 3 and 4). They both subside if the nerve is polarized anodally in the course of their development (see figs. 12 E, 14 D and 15). They both lead to the initiation of propagated spikes if they attain sufficient amplitude and duration.

Besides the fact that the shocks that elicit them differ in polarity, the main differences between them noted in the present study were the following. Although both decrease during the refractory period, the decrease of the cathodal responses is much more striking than that of the anodal responses (fig. 9 A). And whereas a brief cathodal pulse will increase the response to another similar pulse applied shortly afterwards, a cathodal shock given shortly before the end of a long anodal one does not lead to any increase of the anodal response (section I).

These differences are readily explained by the fact that the two responses appear in different conditions: in one instance the response develops while the nerve is depolarized at the cathode, in the other after the nerve has been strongly polarized under the influence of the anode. Thus, the differences in the behavior of the two responses during the refractory period can be attributed to an attenuation of the refractory condition by the anodal pulses, as will be detailed in a later communication. The lack of summation of a cathodal with a following anodal response can in turn be attributed to the subsidence of the cathodal effect brought about by the period of anodal polarization that precedes the anodal response, even if this period is quite brief.

We conclude, therefore, that the two responses are alike, that they are due to a single process and have the same properties (see Arvanitaki, '39, '43a). Any differences between them may be attributed to the fact that they develop in different conditions.

III. The local positive swings. Like the local responses, the postcathodal and the anodal positive swings have several common features. They both develop as a consequence of an inward flow of current through the axon membrane. The two swings may sum, e.g., if an anodal pulse follows a cathodal one. Their time course is approximately the same. They both depress the local responses to cathodal or anodal test shocks.

The present study of the positive swings was not as extensive as that of the local responses. The similarities mentioned above strongly suggest, however, that the two swings, like the two local responses, are alike in nature, that they correspond to the development of the same process (see Arvanitaki, '39, '43a).

IV. The relations between the local responses and positive swings and Lorente de Nó's E_1 potentials. Lorente de Nó ('47) observed potential fluctuations similar to the local responses and positive swings when he applied d.c. pulses to frog nerves and recorded from the cathode or anode or from neighboring extrapolar regions. He assumed that the swings denote the same process that underlies the development of the responses and, therefore, he labeled the 4 waves with the single expression E_1 components of the electrotonic potential.

According to Lorente de Nó the fundamental response of the axon membrane to the passage of current is a reaction that opposes this passage. At the cathode the reaction is a local polarization, which at first opposes the development of the E_1 cathodal depolarization created by the current and later removes this E_1, and becomes manifest during the post-cathodal positive swing at the end of the pulse; at the anode the opposite changes take place, and the local responses would again included the reaction of the nerve.

This interpretation is clearly at variance with that which we have adopted in the present study. Thus, in accord with Hodgkin ('38), we consider the negative variation of the membrane potential that occurs at the cathode at the make of the current to be a local response; Lorente de Nó rejects this view and suggests that the actual response is exhibited by the decline of the wave consequent to the reaction of the membrane. The arguments that follow do not support the latter interpretation.

(a). Conditions that prevent the development of the local responses allow a discrimination between the two hypotheses. Thus, as shown in the diagram in figure 18 A, if the response were the reaction indicated by the line R, its disappearance would lead to the record shown by the broken line E; on the contrary, if the response is the deflection LR, its cancellation would lead to a horizontal record in the conditions in which the present observations were carried out.

As shown in figure 8 C, when the nerve is in the refractory state the disappearance of the cathodal local response leads to a record that approaches the horizontal, that is to a decline of the wave LR, instead of leading to an important increase and prolongation of the negativity developed.

Again, if we apply cocaine to the region tested, the effect is mainly a cancellation of the wave LR (see fig. 17 C), although there is also a slight negative increase toward the end of the pulse. In accord with these observations, Lorente de Nó ('47; fig. VII-3) found that when a nerve is poisoned with ether the cancellation of the local response leads at the pole to the decline of the cathodal wave E_1 and not to an increase of the negativity that develops later.

(b) The interpretation of Lorente de Nó does not allow for a growth of the cathodal local response after a cathodal pulse ceases; the polarization should subside immediately at the end of the pulse. But the studies of Hodgkin ('38), of Rosenblueth and Luco ('50), and the present observations (fig. 12) indicate that if brief cathodal pulses are applied, the local responses reach their maximum some time after the current has ceased flowing. A closely related fact is the greater than linear summation of the responses to two brief cathodal pulses mentioned in section J.

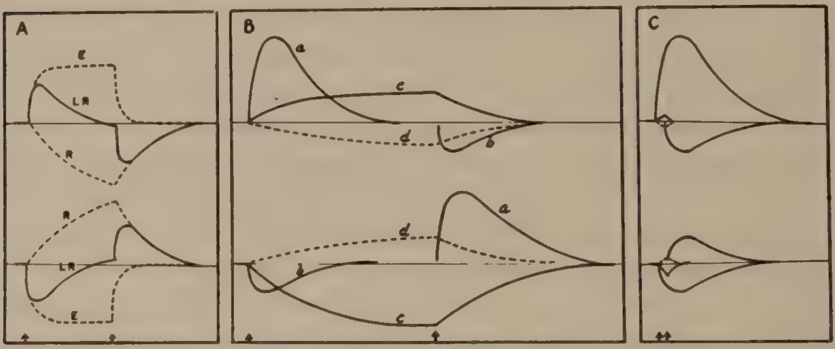

Fig. 18 Diagrammatic representation of some of the processes that develop at the cathode (upper diagrams) and anode (lower diagrams) when d.c. pulses are applied. The arrows denote the beginning and end of the pulses. Upward excursions denote a decrease and lower excursions an increase of the membrane potential.

A Diagram that illustrates Lorente de Nó's ('47) suggestions. E: E_1 electrotonic component; R: reaction of the axon membrane; LR: resultant.

B Diagram showing some of the components encountered in the present study. For explanation see text.

C As in B, but for brief pulses.

(c) Finally, the opposite effects, enhancing or depressing, that a brief anodal pulse may exert on the response to an immediately following cathodal pulse, are not satisfactorily

accounted for by Lorente de Nó's hypothesis. According to his views an anodal pulse should always be followed by an enhancing influence, regardless of its duration or intensity.

V. The measurement of the local responses and positive swings. The reasons for measuring the local responses and swings from the resting base line when the recording electrode *d* is at the position of optimum balance were given in section I. It is clear, however, that this selection involves an arbitrary element. It is also clear that if at each pole different processes with opposite electrical signs develop simultaneously, the measurement, at any given instant, can only indicate the resultant of the summation of the potential fluctuations elicited by those processes. We shall give reasons below in support of the view that at both the anode and the cathode two processes develop simultaneously, and that these processes have an opposite electrical sign and antagonistic effects on the properties of nerves. The measurement of the local responses and positive swings can thus be only relative.

In previous studies (Hodgkin, '38; Pumphrey, Schmitt and Young, '40; Katz, '47; Rosenblueth and Luco, '50) the local response to brief cathodal pulses was measured by adding algebraically the records obtained with these pulses to those resulting from the application of the same pulses with the reversed polarity. The underlying assumption was that the anodal pulses elicited only passive effects.

The present study confirms Lorente de Nó's ('47) criticism that anodal pulses elicit active responses, and that the method of algebraic summation, therefore, is inaccurate. Our observations, however, show that the responses to brief anodal shocks, of subthreshold strength when cathodal, are in general much smaller than the responses to the corresponding cathodal pulses. We may conclude that the error made in the studies mentioned was slight and did not vitiate the inferences drawn.

In connection with the problem of the measurement of the local responses, it is important to consider the question of the relative contribution of the different fibers of a multifibered trunk to the responses recorded, and that of the relative amplitude of the responses at the pole to those recorded some distance away.

Rosenblueth and Luco ('50) showed that the fibers that contribute mainly to the local responses to brief cathodal shocks are the largest myelinated elements. The inference was based on the degree of decline of the responses when more and more fibers of the trunk were made refractory by conditioning threshold stimuli. Using the same test, in the present study we found that the same large, most excitable elements contribute more importantly to the local responses and positive swings than do the finer less excitable fibers.

That the records taken at a short distance from the pole give reliable information on the magnitude of the responses and positive swings that take place at the pole is shown by the measurements illustrated in figures 3 and 7. The decrease of amplitude consequent to increasing the distance from the pole is gradual and the difference between the amplitude at the pole and that at the position of optimum balance is relatively slight.

VI. The dual effects, excitatory and depressing, of the cathode and anode. Under this heading we do not wish to emphasize the well known usual effects of cathodal stimulation, postcathodal depression, anodal depression, and postanodal stimulation or enhancement, but to suggest that the 4 periods may include either excitatory or depressing effects.

Cathodal depression is a recognized phenomenon (see for references Lorente de Nó, '47). In the present observations it was often encountered, overtly or in a latent form. Thus, as shown in figure 11, the initial increase of the local responses to brief test shocks consequent to the application of a long cathodal pulse is followed by a gradual decrease, and the test responses may become smaller than the controls. In addition the degree and duration of the postcathodal depression is determined by the duration of the cathodal pulses employed, if the voltage is maintained constant. It is thus clear that the process responsible for this depression begins to build up during the flow of current.

Excitatory effects during the postcathodal periods are revealed by the greater than linear summation of the responses to two brief cathodal pulses applied in rapid succession (summation of subliminal stimuli; see Pumphrey, Schmitt and Young, '40; Rosenblueth and Luco, '50). They are also revealed by the fact that nerve impulses may arise at the cathode at the break of the current (see Rosenblueth, '41).

Anodal excitatory effects are evidenced by the gradual increase of the test responses that takes place after the initial depression, when test stimuli are applied at increasing intervals after the beginning of a long anodal pulse (fig. 11); the test responses often become larger than the controls in these conditions. Since the postanodal local responses increase with the duration of the shocks (fig. 6) it is plausible that, much as the postcathodal swing is stimulated during the passage of current, the anodal response is also stimulated at the anode during this passage, and that its development is concomitantly prevented by the anodal polarization. Indeed, this double effect is also apparent when an anodal pulse is applied in the course of the development of a local response: the response promptly subsides, but it reappears, and with a larger magnitude, as soon as the pulse ceases. The possibility of eliciting nerve impulses at the anode at the make of a pulse (see Rosenblueth, '41) may, finally, also be cited as evidence for a stimulating anodal influence.

Postanodal depressing effects are revealed by the ability of a brief anodal pulse of moderate intensity to attenuate or cancel the response to an immediately following cathodal pulse (figs. 12 and 13). The cancellation of spike discharge that takes place when a liminal cathodal stimulus is preceded by an anodal shock of appropriate intensity and timing (Blair and Erlanger, '36; Lorente de Nó, '47) is readily explained by this postanodal depression.

These data support the conclusion that both at the anode and at the cathode there develop simultaneously two processes; one that promotes the appearance of local responses, and consequently of nerve impulses, and another that impedes this appearance; and that these processes may outlast the time of application of the pulses. Whether one or the other of the processes predominates at a given moment depends not only on the polarity of the pulses at the tested region, but also on their duration and intensity. Thus, with relatively long cathodal pulses excitation is followed by relative or absolute depression while the pulses flow, and by an even more marked depression when they cease, and the opposite sequence occurs with anodal pulses; but brief cathodal pulses are first followed by excitatory effects, and only later by slight depression, and brief anodal pulses of moderate intensity are immediately followed by depression, and only later by slight excitatory effects.

The diagrams in figure 18 B illustrate the several local processes that appear to develop at the cathode and anode. The upward excursions denote depolarization; the downward deflections indicate an increase of the membrane potential. The waves *a* and *b* are the local responses and positive swings, respectively. Component *c* was seen only in occasional circumstances in the present observations; it will be discussed in a later communication; it may be similar to one of the slow components (E_2 or E_3) of Lorente de Nó ('47).

The component labeled *d* is equivalent to Lorente de Nó's reaction; it represents a counter e.m.f or polarization potential. With the data available it is not clear whether this component denotes an active or a passive process. It has been included in the diagrams because if a counter current is to develop at the end of the pulses some degree of polarization is necessary. In addition, the gradual decay of this component after the application of an anodal pulse could give rise to repetitive discharges. The development of a prolonged anodal local response could also give rise to repetitive discharges. The existence of two different types of opening tetani (see for references Lorente de Nó, '47) is thus explainable.

The main differences between the hypothesis embodied in these diagrams and that proposed by Lorente de Nó (see fig. 18 A) are the inclusion of the local responses among the active processes and the assumption that these responses have a time course of their own. Thus, if a brief pulse is applied, which initiates a cathodal response or an anodal swing, these processes will continue their development after the current ceases to flow (fig. 18 C). This assumption explains postcathodal enhancement and postanodal depression. It also explains why impulses may only be initiated well after the end of brief cathodal pulses. On the other hand, the time course of the responses is influenced by the characteristics of the pulses that elicit them. Thus, the responses to brief cathodal pulses subside more promptly than those to long pulses.

It is possible that the responses and swings may denote changes of opposite sign in the same system or process, as suggested by Lorente de Nó ('47). It is also possible that some of the responses indicated in the diagrams in figure 18 may take place at different double layers in the axon membrane. These suggestions, however, require further study.

The local responses and swings in figure 18 B and C are shown as monophasic. In fact, in the present study the responses were usually followed by a slight positive phase. Arvanitaki ('38, '39a) found the local effects essentially oscillatory, with a variable degree of damping (see also Lorente de Nó, '47; Hodgkin, '48), and showed also that the rate of oscillation increases with the intensity of the currents. If it could be shown that a local response evokes a positive swing, and conversely, that a positive swing can elicit a local response, there would be a possibility that oscillations might develop by this mechanism. In the nerves used in this study, however, the oscillations were highly damped, and the reponses were, therefore, mainly monophasic.

VII. The electrical sign of the excitatory and depressing effects at the cathode and anode. A correlation may be established between the effects mentioned in the previous section and the changes of the membrane potential, as follows.

Whenever the membrane potential decreases, that is, during the development of the local responses, the predominant effects are excitatory, as broadly characterized above, and the intensity of the excitatory effects is approximately proportional to the degree of depolarization. On the contrary, if the membrane potential increases, for instance during the development of the positive swings, depression preponderates, and the degree of depression is roughly correlated with that of polarization (see Arvanitaki, '43b; Lorente de Nó, '47).

It should be emphasized, however, that this correlation is not strict. Thus, if long cathodal pulses are applied, the local response is usually followed by a positive deflection (see fig. 4), yet the responses to test shocks may be greater than the controls when the shocks are applied during this period of increased polarization. Again, if long anodal pulses are delivered, the initial positive swing is usually followed by a period of approximately isopotentiality (fig. 4), but the responses to test shocks, while showing a gradual increase as the shocks are applied later within the pulse, may remain smaller than the controls throughout the period of anodal polarization.

The last observation mentioned suggests that there may be changes of the properties of an axon at a time when the membrane potential does not change, i.e., it suggests that there may occur processes due to the passage of current which alter the axon, but which have no electrical sign.

VIII. The electrical stimulation of axons. The present study confirms the conclusions reached by Rosenblueth and Luco ('50) on the mode of initiation of nerve impulses by electrical stimuli, and amplifies them, as follows.

Whether the nerve impulses arise at the cathode or at the anode, the initiation is always preceded by the development of a local response of sufficient amplitude or duration. A critical amplitude is not required, for shocks of different durations act at quite different levels of the membrane potential (see section H and fig. 10); in addition, with just-threshold pulses the spikes do not start at the peak of the local response, but later, after the response has subsided significantly.

The degree of response necessary for the initiation of impulses varies in different experimental conditions; e.g., larger responses are necessary during the refractory period than those sufficient for the resting nerve. But, at any given time, in multifibered nerves a decrease of the local response results in a decrease of the number of fibers that discharge impulses when the stimuli are liminal, and an increase of response results in the discharge of a large number of fibers.

When the stimuli are anodal the initiation of impulses occurs well after the end of the pulses. Similarly, with weak brief cathodal stimuli the responses always start after the end of the pulses. It is thus clear that the stimuli do not trip the spikes directly; they cause the development of a local response, and if this response reaches threshold, spike initiation ensues.

The local responses, cathodal and anodal, are not spikes that do not develop to the amplitude necessary for propagation. The spike potentials appear in the records as independent phenomena. If the response reaches threshold a spike will suddenly develop, and, when single-fiber spike potentials are identifiable, as they usually are in fine strands of the spinal roots, the responses to successive just threshold stimuli show that the spikes, unlike the local responses, follow the all-or-nothing principle.

Stimulation at the break of an anodal pulse does not differ from stimulation at the make of a cathodal shock. In both instances the initiation of spikes is preceded by the development of a local response, and this response has the same features and properties whether it appears at the anode or at the cathode (see section II). When alternating rectangular currents are applied (figs. 15 and 16), it is impossible to distinguish the anodal from the cathodal responses; the two merge to form a series of single waves that may reach threshold only after several cycles have been delivered.

SUMMARY

A method is described that allows the balancing of the passive effects of d.c. rectangular pulses (figs. 1, 2 and 3), and thus permits accurate recording of the local changes of the membrane potential elicited by subthreshold stimuli in the spinal roots of the cat. These changes include a cathodal and a postanodal negative local response and an anodal and a postcathodal positive swing (fig. 3).

The influence of the following factors on the local responses and swings was studied: the intensity of the pulses (figs. 4 and 5); the duration of these pulses (fig. 6); the distance from the pole (fig. 7); the relatively refractory period (figs. 8 C and F and 9 A); previous or concurrent anodal or cathodal polarization (figs. 11, 12 and 13); local applications of cocaine (fig. 17).

Local responses that outlast spike discharge are described (fig. 9 B). The amplitude of threshold anodal responses to pulses of different durations was studied (fig. 10). The responses to trains of pulses (fig. 14) and to alternating rectangular currents (figs. 15 and 16) were also studied.

In the Discussion the following conclusions are reached. The local responses and positive swings are not due to the rectifying properties of the axon membrane; they denote active changes of the membrane potential (section I). The cathodal and the anodal local responses reveal the development of the same process (section II). The anodal and the postcathodal positive swings are in turn due to the development of a single process (section III). It is possible that the responses and the swings may be changes of opposite sign in the same double layer or process (section VI). Anodal stimulation at the break does not differ in mechanism from cathodal stimulation at the make of a d.c. pulse; in both cases a sufficient amplitude or duration of local response is the necessary and sufficient condition for the initiation of propagated impulses (section VIII).

The relations between the local responses and swings and Lorente de Nó's ('47) E_1 potentials are considered (sections

IV and VI). The intrepretation of the membrane potential changes adopted here differs from that proposed by him (fig. 18).

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RESPIRATORY CHARACTERISTICS OF KANGAROO RAT BLOOD

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ONE FIGURE

This study of the respiratory characteristics of the blood of kangaroo rats was undertaken in order to elucidate a problem arising from the finding that these rodents have an evaporative water loss much lower than that of white rats.

It had been found (Schmidt-Nielsen, '50) that the evaporative water loss of kangaroo rats and pocket mice (Dipodomys spectabilis, Dipodomys merriami, and Perognathus spp., all being rodents of the family Heteromyidae) is only slightly more than half of that of white rats (table 1).

It was not possible to obtain reliable measurements of evaporation from lungs and skin separately. However, since the water loss was of an order that could be expected from the lungs alone, it was assumed that most of the water loss took place as evaporation from the lungs.

Such a low pulmonary water loss posed the problem of whether the lower evaporation was due to:

1. A lower water content of the expiratory air due to lower temperature or to lack of complete saturation with water vapor, or

2. A higher utilization of the alveolar oxygen, requiring less ventilation of the lungs for a given oxygen uptake.

The problem could not be attacked by direct measurements of expiratory air, because mechanical devices, restriction of the animal, and anesthesia changed the evaporation radically.

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It was, therefore, decided to collect evidence in favor or disfavor of the second hypothesis from the respiratory characteristics of the blood.

If the alveolar oxygen should be better utilized than is usual in mammals, e.g. by a reduction of the alveolar oxygen from 21% to 11% instead of to the usual 16%, the alveolar oxygen tension would be so low that the hemoglobin would be fully saturated only if the O_2-dissociation curve of the hemoglobin were shifted to the left as compared with the usual curve for a similar animal (white rat). Such a shift is found in the llama, an animal that regularly breathes the rarified air of high altitudes, and therefore constantly has a low alveolar O_2 tension (Hall et al., '36). Furthermore, a higher oxygen carrying capacity of the blood might offset some of the disadvantages of incomplete saturation of the blood.

TABLE 1

Water evaporation in various rodent species

ANIMAL SPECIES	EVAPORATION, MG H_2O PER ML O_2 CONSUMED
Dipodomys merriami	0.54 ± 0.01
D. spectabilis	0.57 ± 0.03
Perognathus spp.	0.50 ± 0.03
Rattus norvegicus var. alba	0.94 ± 0.03
Mus musculus var. alba	0.85 ± 0.03

Oxygen capacity

The oxygen capacity was determined in whole blood, freshly drawn. The sample was equilibrated with air at 150 to 155 mm O_2 pressure. All equilibrations were made at 37°C. The oxygen content of the blood was determined by Scholander's syringe method (Scholander et al., '47). The equilibration gas was analyzed with Scholander's "Half cc Gas Analyzer" (Scholander, '47). Results were calculated to 760 mm pressure at 0°C.

The oxygen capacity was determined in blood samples from 12 Dipodomys spectabilis and from 5 white rats. The results are presented in table 2.

TABLE 2

Oxygen capacity of blood in kangaroo rat and in white rat

DIPODOMYS SPECTABILIS		RATTUS NORVEGICUS VAR. ALBA	
Weight of animal	Oxygen capacity Ml O_2/100 ml blood	Animal no. (♂♂ 200–250 gm)	Oxygen capacity Ml O_2/100 ml blood
gm			
66	21.0	1	20.4
82	17.3	2	18.7
84	18.8	3	17.6
90	17.2	4	18.7
94	15.5	5	17.6
96	13.7	Average	18.6
101	17.5		
102	19.9		
108	18.4		
108	17.8		
109	18.1		
	14.7		
Average	17.5		

It is clear from these results that the oxygen capacity of kangaroo rat blood is similar to that of the white rat, and that the oxygen transport capacity, therefore, is not different from usual.

Oxygen dissociation curve

The oxygen dissociation curve was determined in whole blood and not on hemoglobin solutions. Equilibrations were made in a glass syringe, rotated in a water bath at 37°C. The analytical methods were the same as those mentioned above.

All values of oxygen content are expressed as per cent saturation. Full saturation (oxygen capacity) is set at 100%.

The partial pressure of carbon dioxide was kept close to 40 mm, with appropriate variations in the oxygen pressure in the tonometer.

Table 3 expresses data for one kangaroo rat and one white rat.

The CO_2 tension in the different equilibrium runs varied somewhat, but not sufficiently to influence the construction of the oxygen dissociation curves for the blood. The dissociation curves are presented in figure 1.

These curves show that the oxygen dissociation curve of the kangaroo rat is close to that of white rats. The difference is such that, in the upper part of the curves, a given saturation might be reached at alveolar oxygen tensions of perhaps 10 mm less in kangaroo rat than in white rat. (The difference might even be less since the CO_2 values are somewhat lower for the kangaroo rat data. CO_2 values like those in the rat would presumably shift the kangaroo rat curve somewhat to the right. If the Bohr effect in kangaroo rat blood is as in other mammals, the two curves are identical within the experimental error.) It is doubtful whether the small difference found can be at all related to the water conservation problem outlined in the Introduction. In any case, the difference is so small that quantitatively it would not permit a utilization of nearly twice as much of the alveolar oxygen, the quantity required if such a phe-

TABLE 3

Per cent oxygen saturation with corresponding values for oxygen tension and carbon dioxide tension

KANGAROO RAT			WHITE RAT		
Per cent O_2-saturation	O_2 tension	CO_2 tension	Per cent O_2-saturation	O_2 tension	CO_2 tension
	mm	*mm*		*mm*	*mm*
100.0	153		100.0	153	
100.0	119	37	100.0	100	43
100.0	100	35	63.7	67	45
97.5	82	37	45.6	55	44
84.0	67	34	31.9	49	40
66.0	58	34			
46.6	54	42			
29.0	41	43			

nomenon were to explain the lower pulmonary water loss in the kangaroo rat.

CO_2 tension in arterial blood

Some information on the composition of the alveolar air could be obtained by analysis of the CO_2 tension in arterial blood. A blood CO_2 tension of the same order as in other mammals would indicate that the composition of alveloar gases is as usual, both with respect to CO_2 and to O_2.

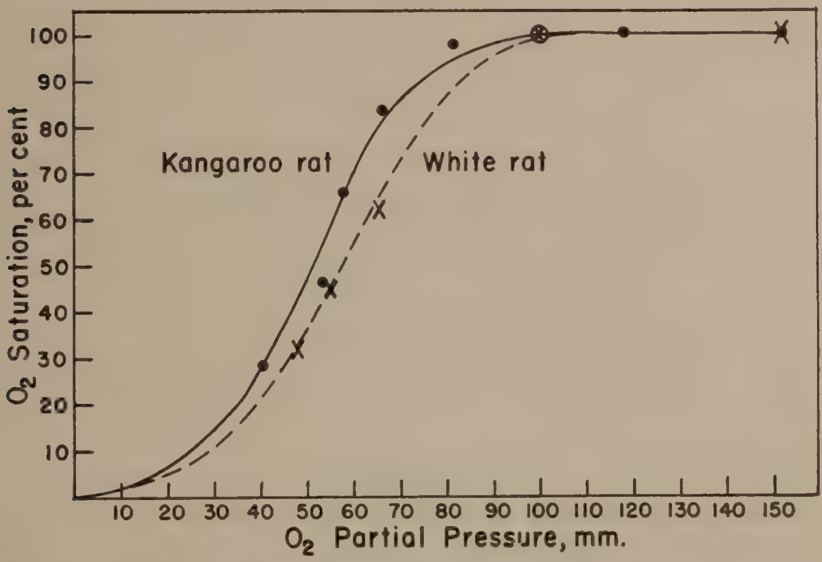

Fig. 1 Oxygen dissociation curves at 40 mm CO_2 for whole blood from kangaroo rat and white rat.

Total carbon dioxide content in venous kangaroo rat blood (heart puncture) was found to be 40.3 ml per 100 ml blood; white rats averaged 41.8 ml per 100 ml venous blood.

The determinations of CO_2 tension in arterial blood were made on animals injected with heparin (1 unit per gram body weight). The blood was taken from the tail arteries by cutting the tail, and collected under paraffin oil at approximately 37°C. A small air bubble equilibrated with the blood sample was then analyzed.

Table 4 shows results which indicate CO_2 tensions so close to the usual values that further investigation was found unnecessary.

TABLE 4

Carbon dioxide tension in arterial blood from kangaroo rat and white rat

DIPODOMYS SPECTABILIS		WHITE RAT	
Animal weight	CO_2 tension	Animal weight	CO_2 tension
gm	*mm*	*gm*	*mm*
106	46.6	130	46.6
104	51.2	144	40.0
102	45.1		

SUMMARY

The respiratory characteristics of blood from kangaroo rat were investigated because of the low pulmonary water loss found in this animal which might be explained by a hypothetical low ventilation of the lungs and a higher utilization of the alveolar oxygen.

This hypothesis was not supported by our findings, which show that the respiratory characteristics of kangaroo rat blood are similar to those of the blood of white rats.

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PHOSPHATASE ACTIVITY IN THE TISSUES OF THE FROG, RANA PIPIENS

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ONE FIGURE

Although there are several reports (Harris, '46; Barth, '47, '50) of phosphatase activity of preparations from frog eggs, there appears to be little known about the phosphatases in the tissues of the adult frog. Ponz ('46, '47) has recently reported the presence of glycerophosphatase activity in the digestive tract, liver, kidney, pancreas, and lung of Rana esculenta. The pH optima obtained by Ponz for the liver phosphatases are similar to those generally reported for the mammalian enzymes.

It is the purpose of this paper to present data on the glycerophosphatase activity of the kidney, liver, spleen, heart, and muscle of the leopard-frog, Rana pipiens. Since information is available on the effect of inorganic ions on mammalian phosphatases it appeared that such information might be used in a comparative manner in studying the frog enzymes. Therefore, the effects of magnesium, manganese, cobalt, zinc, fluoride and cyanide on the alkaline phosphatase of the frog liver were determined. In addition, the tissues were assayed for the phosphoprotein phosphatase described by Harris ('46).

EXPERIMENTAL

The following procedure was used for the determination of the tissue phosphatase activities. Adult specimens of

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Rana pipiens were pithed and the tissue was removed, washed with a small amount of cold distilled water, blotted rapidly on filter paper and weighed to the nearest centigram. The tissue was then homogenized with 10 volumes of cold distilled water, saturated with chloroform, and allowed to stand in the refrigerator for 48 hours. After filtering through a loose cotton plug, 1 ml aliquots of filtrate were added to tubes containing 9 ml of 0.025 M sodium B-glycerophosphate buffered at pH 9.4 and pH 5 with veronal. Incubation was for two hours at 37°C. The reaction was stopped and the proteins precipitated by the addition of 4 ml of 30% trichloracetic acid. Control tubes containing enzyme, buffered substrate, and trichloroacetic acid were prepared for the determination of initial phosphate concentration. The deproteinized tubes were allowed to stand in the refrigerator for one hour and then filtered and a suitable aliquot of the filtrate, usually 0.5 ml or 1 ml, used for the determination of inorganic phosphate. Phosphate was determined by the method of Fiske and SubbaRow ('25) using a Beckman DU spectrophotometer at 660 mμ.

In studying the effects of magnesium, manganese and cobalt on the alkaline liver phosphatase the homogenate was dialyzed against cold distilled water for 5 days and then diluted to 100 ml. Two milliliters of dialyzed homogenate were added to tubes containing B-glycerophosphate buffered at pH 9.4 plus appropriate amounts of magnesium sulfate, manganous chloride or cobaltous chloride to give the desired concentration of activator. Incubation was carried out as before. Control tubes containing no activator were prepared for these determinations.

Undialyzed liver homogenates were used in the experiments involving zinc, fluoride and cyanide. The ions were added in the form of zinc sulfate, sodium fluoride and sodium cyanide.

In determining the effect of heat on liver phosphatase, aliquots of the undialyzed homogenate were preheated in a water bath for one hour. Incubation was at 37°C. and the determinations were run simultaneously with unheated aliquots.

The activities in all cases are expressed as milligrams of phosphorus released per gram of fresh tissue.

Phosphoprotein phosphatase was assayed by the method of Feinstein and Volk ('49) using casein as the substrate and 0.01 M ascorbic acid as the activator.

TABLE 1

Tissue phosphatase activity

Milligrams of phosphorus released per gram of tissue at 37°C.: two hours' incubation

Tissue	pH 9.4	pH 5.0
Kidney	24.33	15.71
	20.57	17.40
	18.98	
Liver	12.85	9.63
	12.88	9.36
	12.38	9.10
	10.05	7.68
Spleen	8.09	10.63
	8.25	10.67
Heart	2.23	4.44
	2.83	4.05
Muscle	0.22	0.35
	0.20	0.36

DISCUSSION

The alkaline and acid phosphatase activities of the various tissues of Rana pipiens are given in table 1 where each value represents duplicate determinations on a single animal. In general, these results are comparable with results obtained from mammalian studies (MacFarlane, '34) in that the kidney and liver exhibit the greatest activity and muscle the lowest. The values obtained for the alkaline phosphatase activity in kidney and liver are also in agreement with Ponz' values for Rana esculenta.

The effects of magnesium, manganese and cobalt on the alkaline phosphatase activity of dialyzed liver homogenates are shown in figure 1. The concentrations of cation are expressed, as suggested by Kay ('31) in terms of "q" values

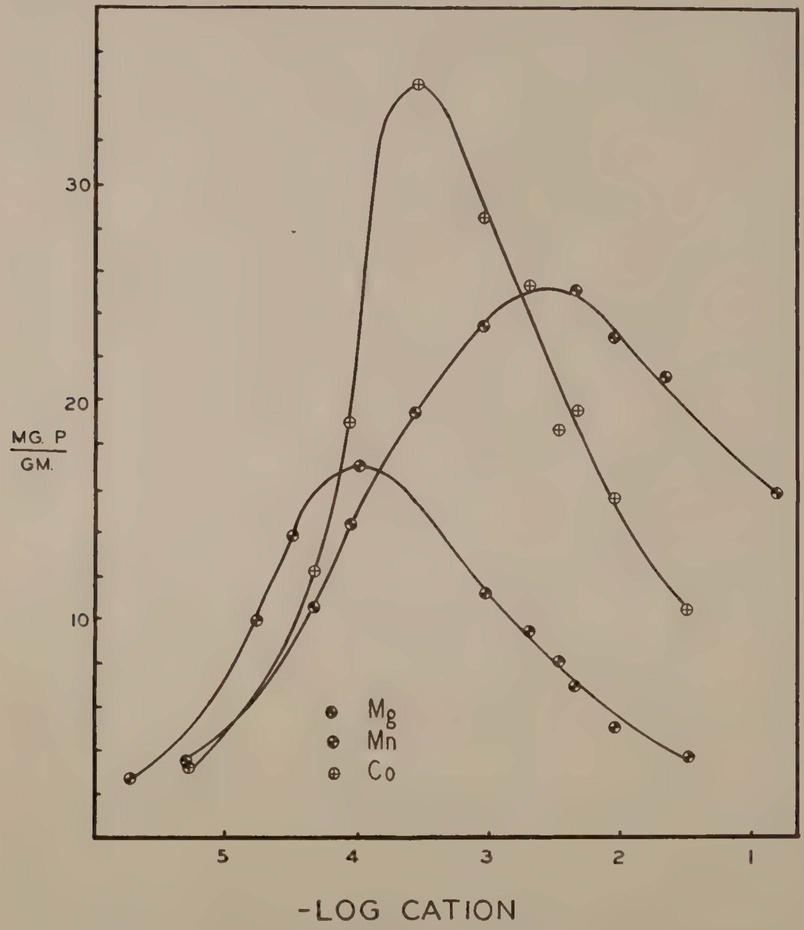

Fig. 1 Effect of Mg^{++}, Mn^{++}, and Co^{++} on frog liver phosphatase at pH 9.4. Incubation for two hours at 37°C.

where $qX = -\log [X]$. These ions are all capable of acting as activators for alkaline phosphatase with cobalt producing maximum activation. The concentrations necessary to produce maximum activation were $qCo = 3.6$, $qMg = 2.5$, and qMn = 4. Similar activation of mammalian liver phosphatase preparations has been reported by Cloetens ('40) and Bamann and Heumüller ('40).

Table 2 shows the effects of zinc, fluoride, and cyanide on the alkaline liver phosphatase. Again these effects are comparable with effects observed on mammalian enzymes. Inhibition of phosphatases by zinc has been reported by Erdtman ('28), Hove et al. ('40) and recently by Bodansky and Blumenfeld ('49). In the study of the effect of zinc on human serum phosphatase Bodansky reports no inhibition at qZn = 5, 10% inhibition at qZn = 4 and 50% inhibition at qZn = 3. The frog liver preparation shows a greater sensitivity to zinc since 9% inhibition occurs at qZn = 5 and 60% at qZn = 4.

TABLE 2

The effect of zinc, cyanide and fluoride on liver phosphatase: pH 9.4

	CONCENTRATION (q)	% INHIBITION
Zinc	5	9
	3	60
Cyanide	2.98	35
	2.70	60
Fluoride	5.74	none
	4.50	none
	2.50	none
	2.0	none
	1.50	

No effect on alkaline phosphatase activity was observed with sodium fluoride over a wide range of concentration. Belfanti et al. ('35) have made similar observations with rabbit liver phosphatase.

Cyanide ion, added as sodium cyanide caused 35% inhibition at qCN = 3 and 60% at qCN = 2.7. Cloetens ('39) reported 95% inhibition at qCN = 2 in a rat liver preparation.

Table 3 shows the effect of preheating the liver preparation for one hour prior to incubation at 37°C. Heating above 55°C. results in almost complete loss of activity.

Phosphoprotein phosphatase. This interesting enzyme, reported in the eggs of Rana pipiens (Harris, '46) appears to be absent in the liver, spleen, heart, and kidney of the adult. Homogenates of these tissues failed to liberate phosphate from casein at pH 6 in the presence of 0.01 M ascorbic acid. Preliminary experiments also indicate that no phosphoprotein phosphatase is present in homogenates prepared from young tadpoles.

This confirms the recent results of Barth and Jaeger ('50). The results may be explained on the basis of the absence of yolk in these preparations since phosphoprotein phosphatase and also phosphotransferase activity have been shown to be localized in the yolk fraction of the frog egg.

TABLE 3

Inactivation of liver alkaline phosphatase by heat. Homogenates held at temperature for one hour prior to incubation at 37°C.

TEMPERATURE	% INACTIVATION
40	12
45	13
50	14
55	25
60	88
70	98

SUMMARY

1. Alkaline and acid phosphatase activity have been determined in the kidney, liver, heart, spleen and muscle of the adult frog, Rana pipiens.
2. The alkaline phosphatase of frog liver is activated by magnesium, manganese, and cobalt; inhibited by cyanide; and not affected by fluoride. The enzyme is rapidly destroyed at temperatures above 55°C.
3. Phosphoprotein phosphatase activity is not demonstrable in the liver, kidney, spleen, or heart.

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COMMENTS
AND
COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

SOME OBSERVATIONS ON THE STRUCTURE OF NERVE IN THE CLAM, MACTRA SOLIDISSIMA

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Casual examination of nerves in the clam, Mactra solidissima, has led several observers, the writer among them, to imagine that this system, in particular the peripheral nerves and connectives, might be of unique interest and value to the neurophysiologist, since at first one is led to suspect the presence of giant fibers. On closer scrutiny this is seen not to be true for the regions of nerve examined. Nonetheless, it seems worthwhile briefly to record the observations made.

There are in this organism, as in others of its group (Brown et al., '50; Hanström, '28) several major ganglia

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[1] This work has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

with interganglionic connectives and peripherally extending nerves. The whole system is contained in a sheath of connective tissue which follows the contours of the ganglia, the nerves and their branches. During life the ganglia and all the nerves are colored bright red. The nature of the pigment is not known, but it may be chemically similar to the yellow pigment which tints the nervous system of another clam, Venus mercenaria. The claim that carotenoids may accumulate in the Golgi apparatus of certain neurones (Helix, Planorbis and Limnaea) is suggestive (Cain, '48). The red pigment is apparently not in the sheath which is seen to be more or less colorless after its neuronal contents have been removed. It would, of course, be interesting to know something of the chemistry of this material and of its functional significance.

Little is known of the functional properties of this nervous system. Tapping on the foot muscle induces a moderately quick, local reflex contraction followed rather slowly by relaxation. Cutting the nerves going into the foot from the viscero-pedal ganglion abolishes this reflex and also decreases the resting tone of the muscle which now becomes much softer to the touch. Electrical stimulation of the nerves produces complex action potentials which by their temporal dispersion indicate the presence of numerous fibers of differing conduction velocities.

Attempts were made to dissect the fresh nerve in sea water and in mineral oil. If the nerve be compressed with the tips of a forceps the contents are displaced to either side of the compressed region while the tubular sheath remains intact. If the nerve be transected the sheath tube remains open at the end. There is no spontaneous efflux of the tube contents under these conditions. If, however, it now be compressed under a cover-slip or with forceps the tube contents can be squeezed out, but they do not disintegrate and flow away. On release of the displacing pressure the contents pull back into the tube to greater or lesser extent. One can also see that the tube contains many small, fiber-like structures whose long axes parallel the long axis of the tube. These are undoubtedly the individual axones. If now one inserts the forceps' tips into the opened sheath it is possible to grasp some of the inside mass. It can be pulled out for a distance, but if it is not broken off it tends to retract into the tube upon release of the forceps. If the sheath be opened longitudinally one can attempt to tease out single axones. Whereas, however, such teasing apart of axones is relatively easy and reveals clear-cut interaxonal cleavage planes in such nerves as those of mammals, frogs, crabs and squid, no such clear separation is possible in Mactra. There do not appear to be clear cleavage planes.

These observations thus showed the nerve to be surrounded by a rather tough sheath, to contain elastic structures, and to have its component elements so surface adherent or so firmly bound together in some other way as to be, under the conditions extant, inseparable without tearing.

Samples of nerve were then fixed in Zenker-Formol solution for 6 hours. Following this the material was kept at 37°C. for 48 hours in saturated potassium dichromate, was then washed for 24 hours in running tap water, was then kept in iodine alcohol for 48 hours and was finally stored in 80% alcohol containing calcium and cadmium chloride. Paraffin sections were then mounted with albumin and stained with Masson stain.

Such sections show the tubular sheath to be made up of concentric or intertwining layers of connective tissue fibers. Inside the sheath, but not uniformly distributed, one sees many axones of varying size. In the spaces between them there is a densely matted material of a fibrillar nature and containing many densely staining granules. Beneath the tubular sheath and distributed more or less randomly among the axones there are other cells with granular cytoplasm and with large, densely granulated nuclei. The axonal cytoplasm (axoplasm) itself shows a faint stippling or granularity. These latter granules are seen at a magnification of 970 × but not at 450 ×. One wonders whether any or all of the granules seen might be pigmentary and confer the red color on the living nerve. Unfortunately the axoplasmic granules are too small, even if they exist in the living axone, to be sought for in vivo and to be used to give an index of viscosity in terms of Brownian motion.

ACKNOWLEDGMENTS

It is a pleasure for the writer to acknowledge his indebtedness to Mr. Robert Barnett of the University of Chicage Department of Physiology for calling his attention to Mactra as a potentially interesting form, to Dr. Stuart W. Smith of the University of Colorado Department of Anatomy for fixing the tissues for him, and to Mr. John Boldosser of the University of Vermont for cutting certain of the sections. Dr. S. W. Kuffler also participated in the observations.

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THE INFLUENCE OF THE AXON SHEATH ON PHOSPHATE DIFFUSION

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ONE FIGURE

Because of the considerable interest currently being shown the question of whether the connective tissue sheath of the frog axon is a factor in controlling the diffusion of solutes into the fibre, it seems worthwhile for us to report briefly some of our observations on the penetration of the radioactive phosphate into the frog Sciatic nerve. In these experiments, paired nerves were used, one being subjected to dissection in order to remove the connective tissue sheath and the other retained as a control. Both nerves were kept in frog Ringer solution aerated with 95% O_2 and 5% CO_2 for several hours and tested periodically for conduction. At the end of a two or three hour period at 20°C. both nerves were transferred to radioactive Ringer solution containing 0.1 mM HPO_4 with P^{32} and the uptake of the radioactivity measured. The results obtained are shown in figure 1 and represent 5 experiments of the sort outlined above. All points have been corrected for the amount of P^{32} in the extra-cellular space. This correction is not a large one, since during the experimental period the

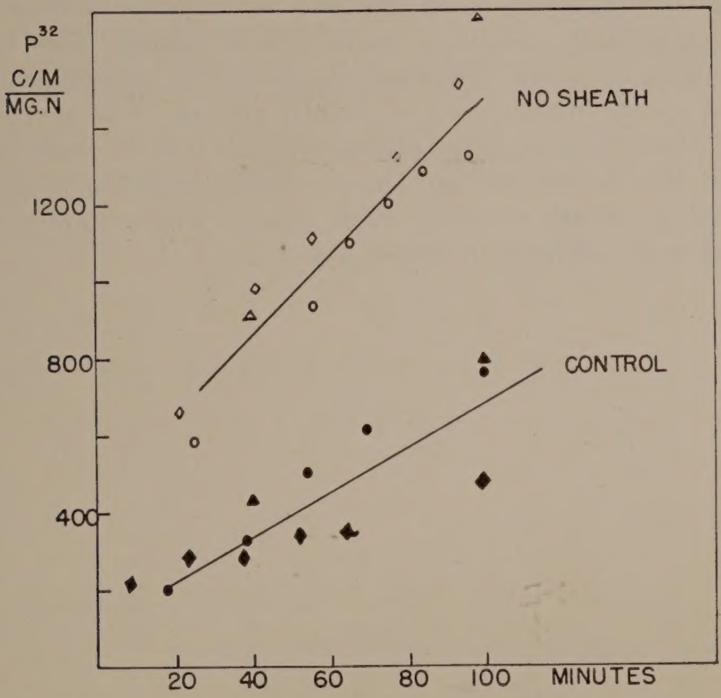

Figure 1

nerves accumulated phosphate to a value several times that of the external solution.

It is difficult to interpret the data in terms of the physico-chemical mechanism involved because there are two effects which come into play with the removal of the nerve sheath, (a) the removal of the sheath, which may itself be a barrier to diffusion, and (b) the expansion of the nerve which gives the individual fibres more distance between themselves and hence a greater volume of extra-cellular space. This latter mechanism in effect, decreases the diffusion resistance occasioned by the fibres being so tightly packed in a nerve and hence allows a more rapid phosphate diffusion throughout the nerve. We cannot rule out the possibility that the fibres are injured by sheath removal. Such injury, however, is not reflected in any change in action potential.

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